

# Leukemia Stem Cell Fates are Determined by DNA Methylation Levels

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# I Abstract

DNA methylation is one of the major epigenetic processes which is crucially involved in orchestrating gene regulation primarily by repression of gene expression. It has been shown that DNA methylation plays an important role in controlling functional programs of embryonic and tissue stem cells. As altered DNA methylation patterns are a hallmark of cancer, we hypothesized that DNA methylation might be equally important for cell fate determination of cancer stem/initiating cells (CSC). To test this, I analyzed a genetic knockdown mouse model of the main somatic DNA methyltransferase *Dnmt1* in the context of three different oncogene driven leukemia models.

A bilinear B-lymphoid/myeloid leukemia model was utilized to test the role of DNA methylation in lineage decision processes of a bi-potential leukemia stem/initiating cell (LSC). Whereas hypomethylated LSCs were capable to form a myeloid leukemia, no B-lymphoid blasts were given rise to by these cells. Moreover, failure of hypomethylated cells to develop T-cell lymphomas in a *Notch1*-based leukemia model demonstrated their profound lack of T-lineage commitment capacities. These data suggest that lineage fate choices of LSCs are determined by the level of DNA methylation. Furthermore, the effect of hypomethylation on the acquisition and maintenance of leukemia self-renewal potential was investigated in a myeloid leukemia model. Both *in vitro* and *in vivo* assays revealed a severely reduced self-renewal potential of transformed *Dnmt1* knockdown cells. This was illustrated by a more than 10-fold reduction of functional LSCs in hypomethylated leukemias. However, contrasting the drastic cell-intrinsic impairments of LSC function by reduced DNA methylation, leukemia development was found to be unaffected by hypomethylated bone marrow stroma. Mechanistically, treatment of cell lines with a demethylating drug led to enhanced expression of differentiation factors due to loss of methylation mediated gene silencing. This was followed by inhibition of leukemia cell growth, thus providing a potential mechanism for impaired functions of hypomethylated leukemias. Collectively, this thesis revealed a critical role for DNA methylation levels in malignant self-renewal and lineage fate choices. These new insights into epigenetic regulation of CSCs suggest that epigenetic therapy displays a potential treatment concept specifically targeting CSCs.

Keywords: DNA methylation, cancer stem cell, self-renewal, lineage fate choice

## II Zusammenfassung

DNA Methylierung ist ein zentraler epigenetischer Prozess, welcher, hauptsächlich durch Repression von Genexpression, entscheidend an der Organisation von Genregulation beteiligt ist. Dieser Vorgang ist wichtig für die Funktion sowohl von embryonalen als auch von Gewebs-Stammzellen. Krebszellen weisen häufig veränderte DNA Methylierungsmuster auf, was auf eine ähnlich wesentliche Rolle der DNA Methylierung bei Zellschicksalsentscheidungen von Krebsstammzellen hindeutet. Diese These wurde hier mit Hilfe eines Mausmodells mit verringerter Expression der DNA Methyltransferase *Dnmt1* anhand drei verschiedener Leukämie Modelle untersucht.

In einem bi-linearen B-lymphatischen/myeloischen Leukämie Modell konnte gezeigt werden, dass hypomethylierte, bi-potente leukämieinitiierende (Stamm-)zellen (LSZ) myeloische Krebszellen hervorbringen, allerdings nicht zur Bildung von B-lymphatischen Leukämiezellen befähigt sind. Darüber hinaus konnte in einem T-Zell-spezifischen Leukämie Modell gezeigt werden, dass reduzierte *Dnmt1* Expression nicht mit der Bildung von T-Zelllymphomen vereinbar ist. Detaillierte Analysen eines myeloischen Leukämie Modells ergaben, dass LSZs mit verringertem DNA Methylierungsgrad ein vermindertes Selbsterneuerungspotenzial aufweisen, was an einer um mehr als zehnfach geringeren Zahl funktioneller LSZs deutlich wurde. Im Gegensatz zu den starken Einschränkungen im Funktionsrepertoire von LSZs durch verminderte *Dnmt1* Expression, hatten hypomethylierte Knochenmarks-Stromazellen keinen Effekt auf die Entwicklung von Leukämien. Außerdem konnte gezeigt werden, dass Behandlung verschiedener leukämischer Zellen mit demethylierenden Agenzien zu einer teilweisen Aufhebung methylierungsvermittelter Genrepression führte. Die dadurch verstärkte Expression von Differenzierungsfaktoren verminderte das Leukämiewachstum, was einen möglichen Erklärungsansatz für das eingeschränkte Potenzial hypomethylierter Leukämien darstellt.

Die Ergebnisse dieser Arbeit demonstrieren eine zentrale Rolle der DNA Methylierung für die Selbsterneuerung und Linienwahl von LSZs, und erlauben somit neue Einblicke in die epigenetische Regulation von Krebsstammzellen. Diese Erkenntnisse implizieren, dass Krebsstammzellen möglicherweise ein geeignetes Ziel für epigenetische Therapieansätze darstellen.

Schlagwörter: DNA Methylierung, Krebsstammzelle, Selbsterneuerung, Linienwahl

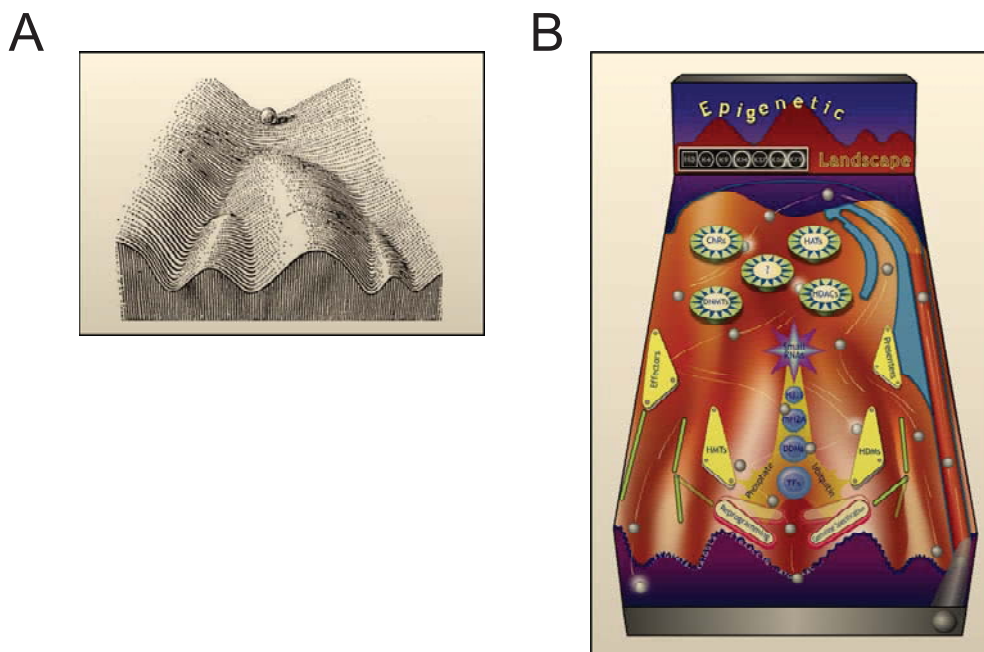
# 1 Introduction

## 1.1 Epigenetics

In 2001 the sequencing of the human genome had been essentially completed, displaying a milestone in molecular biological research [Lander, et al., 2001; McPherson, et al., 2001]. Even though the genome is the ultimate template of our hereditary, today's understanding is that the knowledge of the primary DNA sequence itself is merely the foundation for understanding how the genetic program is read and implemented. Research in the last years has revealed increasing importance of information which is “outside” or “above” genetics, or in another word “epigenetic”. The term epigenetics was coined by Conrad H. Waddington in 1942 as a fusion of the words genetics and epigenesis describing “the branch of biology which studies the causal interactions between genes and their products, which bring the phenotype into being” [Waddington, 1942]. Epigenetics is the study of those processes by which the genetic information, defined as genotype, interacts with the environment in order to produce its observed characteristics, defined as phenotype. This offers a conceptual model of how the phenotype is produced through the interaction of genes with their surrounding without any changes in the underlying DNA sequence, consequently representing a bridge between genotype and phenotype. Over time, a variety of epigenetic processes has been described, like imprinting of maternal or paternal genes [McGrath and Solter, 1984; Surani, et al., 1984], X chromosome inactivation [Lyon, 1961] or paramutation in maize [Brink, 1958], which will not be addressed in more detail here, as they are out of the scope of this work.

More important here, cellular differentiation processes are regarded as epigenetic phenomena. Even though cells of a multicellular organism share the same genetic instruction sets, a great diversity of cell types with very different terminal phenotypes is generated from the originally totipotent cell. During this development the cell undergoes changes in its epigenetic state, a fact that has been famously illustrated as the epigenetic landscape by Conrad H. Waddington in 1957 [Waddington, 1957]. The epigenetic landscape (**Figure 1A**) is a metaphor displaying the process of cellular decision-making, with a marble (representing a cell) rolling down a hill into

one of several valleys. The cell can follow different permitted trajectories, finally reaching its destination at the bottom of a certain valley, reflecting a terminally differentiated state. From today's point of view, we know that at each point in this slope the cell has a specific epigenetic state which is causal for the cell's gene expression profile. Thus, the epigenetic information of a cell (epigenome) displays a stable and heritable, yet changeable, layer of information which instructs cell fates by defining the activity of genes. This is achieved by epigenetic alterations which regulate both chromatin structure and the accessibility of the DNA. Our current knowledge about such epigenetic modifications and players led to an updated version of Waddington's landscape transforming it into a pinball map (**Figure 1B**; [Goldberg, et al., 2007]). Countless mechanisms involving effectors, players and presenters have been identified in years of intensive research, some of which will be introduced in the following section.



**Figure 1: Waddington's epigenetic landscape evolving to a pinball map**

**A)** In the epigenetic landscape, a cell, represented by a marble, faces a number of branching points on its way down the hill of cellular development, eventually reaching one of the valleys, representing potential phenotypic endpoints [Waddington, 1957]. **B)** In this modern version of Waddington's picture the landscape has transformed into a pinball map. Many structures and actors push and redirect the pinball (cell) and guide it along the correct way to the desired endpoint. Illustrated by Sue Ann Fung-Ho [Goldberg, et al., 2007].



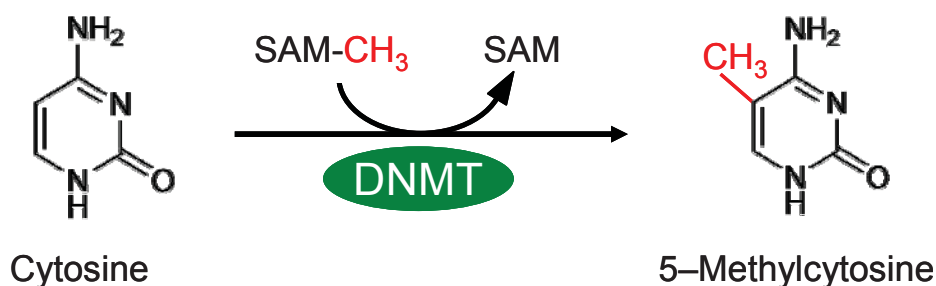
At large, epigenetic modifications fall in two main categories: DNA methylation and histone tail modifications. The nature of those modifications defines the overall structure of the chromatin - the complex of DNA and its associated proteins. The state of chromatin architecture, in turn, determines the accessibility to the underlying DNA, consequently regulating transcriptional activity.

At the heart of chromatin structure conserved histone proteins act as building blocks for packaging DNA into nucleosomal repeats [Strahl and Allis, 2000]. The unstructured tails of the histone proteins can be equipped with different kinds of modifications, such as acetylation, methylation, phosphorylation or ubiquitination, which are placed at specific positions of the amino-terminal tail. For example, whereas methylation of histone H3 lysine 4 (H3K4) is generally associated with transcribed chromatin, methylation of H3K9 or H3K27 usually correlates with repression [Bernstein, et al., 2007]. Histone modifications are added by catalytic enzymes which serve as writers, e.g. the histone methyltransferase SUV39H1, and the mark is recognized by a reader or an effector (like HP1 proteins), which launches the biological implementation at this specific locus [Lachner, et al., 2001]. The sum of all histone modifications is thought to be deciphered as a histone code installing an epigenetic state which determines the actual readout of the genetic information of a certain locus through activation or silencing [Jenuwein and Allis, 2001].

Apart from the just recently discovered 5-Hydroxymethylcytosine [Kriaucionis and Heintz, 2009; Tahiliani, et al., 2009], DNA methylation is the only known covalent modification of DNA in mammals. As it is a key aspect of this thesis it will therefore be introduced more thoroughly in the next chapter.

## 1.2 DNA methylation

DNA methylation describes the addition of methyl groups to the DNA and is found both in prokaryotic and eukaryotic organisms, including fungi, plants, non-vertebrates and vertebrates. Some species are devoid, or almost completely devoid of DNA methylation, like *Caenorhabditis elegans* or *Drosophila melanogaster* [Bird, 2002]. In vertebrates DNA methylation occurs almost exclusively at cytosine residues in the context of a CpG dinucleotide. As depicted in **Figure 2**, DNA methyltransferases (DNMTs) catalyze the transfer of a methyl group from S-adenosylmethionine (SAM) to the C5 position of a cytosine.



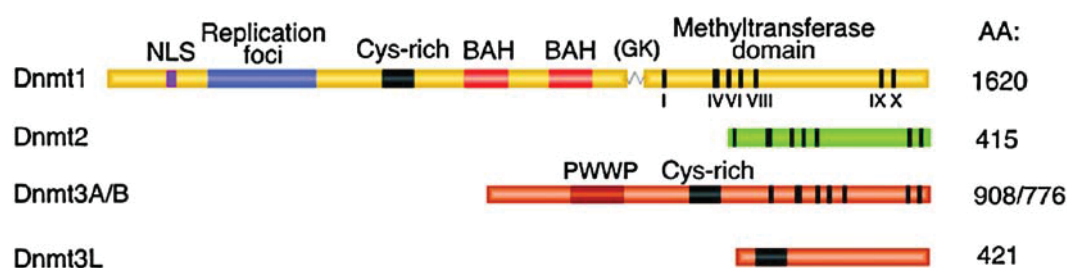
**Figure 2: Cytosine methylation catalyzed by DNA methyltransferase (DNMT)**

A methyl group (-CH<sub>3</sub>) is added to the carbon-5 position of a cytosine residue of the DNA. S-adenosylmethionine serves as methyl group donor. The reaction is catalyzed by DNA methyltransferases (DNMTs).

Cytosine residues are hotspots of base substitution mutations as they are vulnerable to spontaneous deamination. Deamination of an unmethylated cytosine yields uracil, a base which is removed from the DNA sequence by the enzyme uracil glycosylase [Lindahl, 1974]. In contrast, deamination of 5-methylcytosine produces thymine, a normal DNA base, which is hence not removed by any DNA repair machinery eventually causing a G-C to A-T pair transition [Coulondre, et al., 1978; Lindahl, 1982]. As an evolutionary consequence, this CpG hypermutability caused an approximately 5-fold underrepresentation of this dinucleotide throughout the genome [Bird, 1980; Lander, et al., 2001]. 55-90 % of CpGs in the vertebrate genome are methylated and methylation is mainly found in transposable elements and endogenous retroviruses [Bird, et al., 1985; Yoder, et al., 1997b]. However, unmethylated CpG-rich regions are found in the genome: In these so called CpG islands CpG sites occur at the frequency expected by base composition and they are primarily found at the 5' ends of genes [Bird, 1986; Gardiner-Garden and Frommer, 1987]. DNA methylation was shown to be involved in multiple functions like transcriptional silencing, heterochromatin formation, genomic stability, silencing of endogenous retroviruses, genomic imprinting and X chromosome inactivation [Goll and Bestor, 2005; Jaenisch, 1997]. Before discussing the biological functions of DNA methylation in respect to its role in development and disease in the chapters 1.3 to 1.5, the following part will introduce the actors involved in the establishment and translation of the methylation pattern.

### 1.2.1 Establishment and maintenance of the methylation system

During mammalian development, the DNA methylation pattern is subject to dramatic changes which are managed in a highly regulated process [Monk, et al., 1987]. During cleavage, first, DNA methylation is actively stripped off the paternal genome, followed by passive erasure of maternal methylation marks in the following cell divisions [Li, 2002; Mayer, et al., 2000; Oswald, et al., 2000; Rougier, et al., 1998]. Between implantation and gastrulation a wave of global methylation reestablishes the overall methylation pattern which is maintained in the somatic cells throughout life [Jaenisch, 1997]. These facts imply the existence of two general classes of DNA (cytosine-5) methyltransferases (DNMTs), a prediction which had already been made 35 years ago [Holliday and Pugh, 1975; Riggs, 1975]: A class of *de novo* enzymes responsible for the establishment of methylation patterns at specific sequences in early development and a group of maintenance enzymes preserving the methylation pattern during cell division. Up to date, five mammalian DNMTs are known which can be placed in three different groups based on their structural and functional characteristics (**Figure 3**, [Goll and Bestor, 2005]).



**Figure 3: Murine DNMT family members**

Protein length of the five DNMTs is indicated on the right side (aa, number of amino acids). The regulatory N-terminal domain contains a number of functional motifs: a nuclear localization signal (NLS), a sequence required association with the replication foci, a cysteine-rich zinc finger DNA-binding motif and bromo-adjacent homology (BAH) domains, a protein-protein interaction module targeting DNMT to the replication foci. The PWWP tetra-peptide is essential for DNMT binding to chromatin. The C-terminal part harbors the catalytic methyltransferase domain with six conserved motifs [Goll and Bestor, 2005]

DNMT2 was assigned to the DNMT family due to structural homologies, yet it was found that it does not possess any DNA methyltransferase activity. Instead, it was discovered to be an RNA methyltransferase, methylating cytosine 38 in the anticodon

loop of tRNAs [Goll, et al., 2006; Schaefer, et al., 2010; Yoder and Bestor, 1998]. Due to DNMT2 actually being falsely categorized as a DNMT, it will not be discussed any further in this chapter. The maintenance methyltransferase DNMT1 and the DNMT3 family, responsible for de novo methylation, will be introduced in the following section.

#### *1.2.1.1 DNMT1*

The gene encoding the 1620 amino acids long protein DNMT1 is located on chromosome 9 of the mouse genome and displays the first eukaryotic methyltransferase to be cloned and purified [Bestor, et al., 1988]. A 5- to 30-fold substrate-preference for hemi-methylated DNA compared to un-methylated DNA confirmed its function as maintenance methyltransferase [Gruenbaum, et al., 1982; Yoder, et al., 1997a]. During the S-phase of the cell cycle DNMT1 is recruited to the regions of active DNA replication, the replication foci, mediated by sequence motifs in the N-terminal part of DNMT1. A specific motif interacts with the proliferating-cell-nuclear-antigen (PCNA), the DNA clamp protein, which is located at the replication forks during DNA replication [Chuang, et al., 1997; Leonhardt, et al., 1992]. Besides PCNA, several other factors have been shown to interact with DNMT1 and help its recruitment to the replication forks. For example, UHRF1 (ubiquitin-like, containing PHD and RING finger domains 1) appears to tether DNMT1 to the chromatin by directly interacting with DNMT1. The SRA (SET and RING associated) domain of UHRF1 was shown to strongly preferentially bind to hemimethylated DNA, being at least in part responsible for DNMT1's specificity for its physiological substrate [Bostick, et al., 2007].

The methyltransferase domain of DNMT1, harbored at the C-terminal part of the protein, catalyzes the transfer of a methyl group to the nascent DNA strand if a methyl group is present on the parallel position on the parental strand [Gruenbaum, et al., 1982]. Thus, DNMT1 passes on the epigenetic information during cell generations by faithfully copying the parental strand methylation pattern onto the progeny DNA strand.

### 1.2.1.2 The DNMT3 family

In 1996, En Li's group generated a null mutant of the so far only known DNA methyltransferase, DNMT1, and the mutant embryonic stem (ES) cells still showed low but stable levels of methyltransferase activity, suggesting the existence of a second mammalian methyltransferase [Lei, et al., 1996]. Two years later, the closely related DNMT3a and DNMT3b were identified and found to be crucial for *de novo* methylation but not to be involved in the maintenance of DNA methylation [Okano, et al., 1999; Okano, et al., 1998].

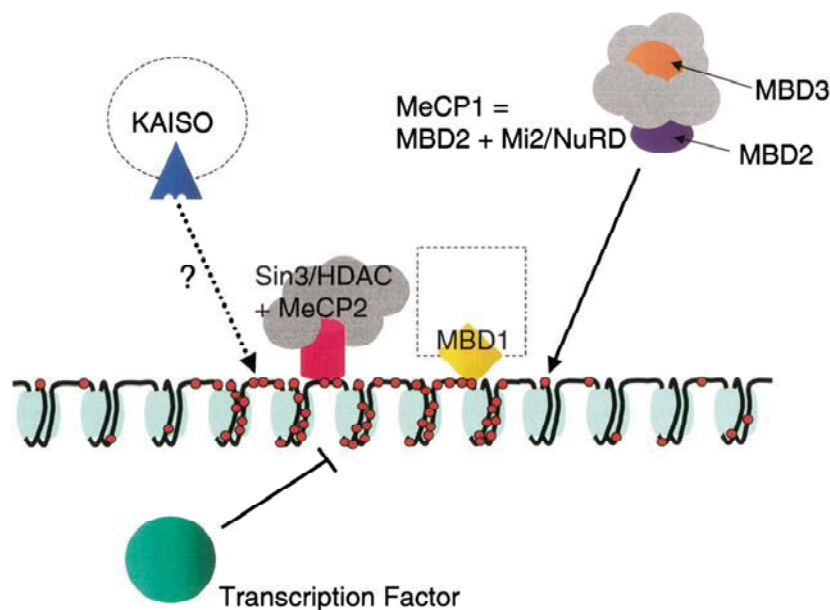
Next to DNMT3a and DNMT3b, the DNMT3 family comprises a third member: the DNMT3-like protein, DNMT3L [Aapola, et al., 2000]. Even though Dnmt3L is devoid of enzymatic methyltransferase activity, it was shown to be essential for the establishment of maternal genomic imprints by interacting with Dnmt3a [Bourc'his, et al., 2001; Hata, et al., 2002; Jia, et al., 2007]. Furthermore, recent findings indicated an interesting link of DNA methylation to histone modifications. DNMT3L was found to bind unmethylated, but not methylated, histone 3 lysine 4 (H3K4), suggesting a specific recruitment of *de novo* methyltransferases to unmethylated H3K4, which might display a mechanism for transmitting non-inheritable histone marks by means of heritable DNA methylation [Ooi, et al., 2007].

Gene silencing at specific loci has been shown not to be exclusively exerted by the DNA methyltransferases, but rather is an interplay between different epigenetic silencing mechanisms, which ensures a reliable shutdown of promoter sequences. Even though it is still largely unclear which mechanisms orchestrate the interactions between epigenetic processes, some connections between epigenetic factors have been uncovered in the last years, starting to shed some light on the epigenetic networks. In 2006, DNMT1, DNMT3a and 3b were all shown to interact with the Polycomb group (PcG) protein EZH2 (Enhancer of Zeste homolog 2), which functions as a histone methyltransferase in transcriptional repression. This interaction serves as a mechanism to dictate site specific DNA methylation at certain loci guaranteeing proper establishment and maintenance of gene silencing [Vire, et al., 2006]. One year later, the connection between the H3K9 methyltransferase G9a and DNMT1 was uncovered providing a mechanism by which DNA methylation activity is recruited to specific targets [Smallwood, et al., 2007]. This further emphasizes the

interdependency of different processes to allow epigenetic repression in a targeted fashion.

### 1.2.2 Translating DNA methylation marks

DNA methylation is usually associated with transcriptionally silent chromatin. But how does DNA methylation interfere with transcription? Two models of repression can be envisaged for this process, both of which have been shown to be biologically relevant (**Figure 4**, [Bird, 2002]).



**Figure 4: Transcriptional repression mechanisms by DNA methylation**

Two basic models for transcriptional silencing by DNA methylation have evolved: In the first, DNA methylation (red circles) can directly repress transcription by preventing transcription factors from binding to their cognate DNA sequences. In the second model, transcriptional inhibition is achieved by specialized Methyl-CpG-binding proteins (MBPs), like MBD1-3, MeCP2 and Kaiso, which recruit different chromatin modifying repressor complexes [Bird, 2002].

In the first model, transcriptional activators are directly prevented from binding to their cognate sequences in regulatory DNA elements. Many factors are known to bind to CpG-containing sequences and some of them fail to bind the DNA if the cytosine residue is methylated. One of the most prominent examples for this mode of action is the reduced binding of the oncoprotein c-Myc to E-box sequence motifs by methylating the CpG within this recognition site [Prendergast and Ziff, 1991]. More recently, the chromatin organizer CTCF was shown to bind in a methylation sensitive fashion

to the non-methylated maternal allele of *H19/Igf2* locus in mouse. Thus, CTCF can exert its insulating function, meaning it is shielding enhancer elements, to specifically silence the maternal copy of the *Igf2* gene, whereas the methylated paternal copy prevents CTCF binding, consequently allowing surrounding enhancers to drive *Igf2* expression [Bell and Felsenfeld, 2000; Hark, et al., 2000; Holmgren, et al., 2001; Szabo, et al., 2000].

The second repression mechanism involves specialized proteins, so called Methyl-CpG-binding proteins (MBPs), which bind to methylated CpG sites and mediate silencing of gene expression by targeting co-repressors to the respective loci. The family of MBPs comprises six members (MBP1-4, MeCP2 and Kaiso) and all but MBP3 and Kaiso possess a conserved Methyl-CpG-binding domain (MDB) to bind methylated DNA [Hendrich and Bird, 1998]. Bound MBPs recruit repressor complexes with chromatin remodeling or modifying properties, like MeCP2 recruiting a histone deacetylases (HDACs) together with the co-repressor Sin3 [Jones, et al., 1998; Nan, et al., 1998]. Repressing mechanisms via MBP1 are not entirely clear yet, but MBP1 was shown to be associated with H3K9 histone methyltransferase SETDB1 [Sarraf and Stancheva, 2004]. Another example for a repressor complex is MeCP1, which comprises MBP2, MBP3, the HDAC complex NuRD and Mi-2, a chromatin remodeling protein from the SWI2/SNF2 family [Wade, et al., 1999; Zhang, et al., 1999]. Finally, Kaiso, which recognizes methyl-CpG with its zinc-finger domain, is known to mediate transcriptional silencing by recruiting the HDAC-containing co-repressor complex N-CoR [Yoon, et al., 2003].

More recent studies have uncovered a further mechanism by which transcription is suppressed by DNA methylation. In this case, instead of inhibiting transcriptional initiation by promoter methylation, transcriptional elongation efficiency is decreased by reducing Pol II processivity through methylation of intragenic CpGs [Lorincz, et al., 2004].

### 1.2.3 Removal of DNA methylation marks

Even though DNA methylation is a stable epigenetic mark, it is yet known to be reversible, which raises the question about processes erasing the methylation marks from cytosines. The most obvious solution is a passive loss of methylation during cell division, when the parental methylation mark is not copied to the daughter strand.



However, loss of DNA methylation in the male pronucleus of the zygote (see 1.2.1, page 10) occurs without cell division and corresponding DNA replication, suggesting an active removal, rather than a passive loss. The process of active demethylation in mammals is yet only poorly understood. One possible scenario would be the removal of the methyl group, which is unlikely as the carbon-carbon bond is thermodynamically stable [Bird, 2002]. Furthermore, demethylation could be accomplished by a DNA repair-like process, such as base excision repair or nucleotide excision repair, but the role of these mechanisms in mammalian demethylation has been only insufficiently described [Wu and Zhang, 2010]. Recent studies suggested Gadd45a (growth arrest and DNA-damage-inducible protein 45 alpha) as a key regulator of active DNA demethylation through promoting DNA methylation [Barreto, et al., 2007]. Demethylation in plants, in contrast, is much better understood. Plants use DNA glycosylases, normally associated with DNA repair, to remove 5-methylcytosines and replace them with cytosines [Gong, et al., 2002]. However, in mammals and animals in general, factors acting as active demethylases have not yet been compellingly identified, leaving the question for active DNA demethylation mechanisms a topic of current research.

### **1.3 DNA methylation in development and differentiation**

With DNA methylation being a crucial part in gene regulation, great research effort has been put into experimental studies aiming to uncover the role of DNA methylation in developmental and differentiation processes. For this purpose, DNA methyltransferase activity was manipulated experimentally by either directly targeting the genes encoding factors involved in DNA methylation processes or by pharmacological inhibition with demethylating reagents.

Targeted ablation of methyltransferase genes in mouse models yielded crucial insights into the function of DNA methylation. Several mouse models using different knockout alleles of the *Dnmt1* gene have been generated to examine the role of the maintenance methyltransferase *in vivo*. All knockout mice showed profound demethylation causing embryonic lethality between day 8.5 and 10.5 p.c. [Lei, et al., 1996; Li, et al., 1993; Li, et al., 1992].

Indispensability was also shown for the *de novo* methyltransferases *Dnmt3a* and *Dnmt3b*. While *Dnmt3a*-deficient mice survive to term, but are runted and die in early



adulthood with defects in the intestinal system and in spermatogenesis, *Dnmt3b* knockout mice die around day 14.5 of embryogenesis due to growth impairment and neural tube defects [Okano, et al., 1999]. Combined deficiency of *Dnmt3a* and *Dnmt3b* leads to even more severe defects than the single mutants with growth and morphogenesis arrest causing death around day 9.5 [Okano, et al., 1999]. Taken together, these studies showed that all *Dnmt* family members are indispensable for embryonic development and the severity of the observed phenotypes strongly underlines the importance of DNA methylation in early developmental processes.

Besides the importance of DNA methylation in embryogenesis, a crucial role for this epigenetic process has also been shown in several somatic tissues. Due to the embryonic lethality caused by the deletion of *Dnmt1*, a conditional knockout allele (*Dnmt1<sup>lox</sup>*) was constructed, where the catalytic domain, harbored in exons 4 and 5, was flanked by loxP sites, allowing the inducible and cell-type specific deletion of *Dnmt1* with the Cre/loxP system [Jackson-Grusby, et al., 2001; Sauer and Henderson, 1989]. Deletion of *Dnmt1* in fibroblasts from homozygous *Dnmt1<sup>lox</sup>* mice with a Cre-recombinase expressing retrovirus led to severe demethylation, causing re-activation of silenced endogenous retroviral elements and dramatic changes in gene expression, finally leading to p53-dependent cell death.

The adult brain exhibits higher levels of DNA methylation than any other tissue and DNA methylation levels were shown to undergo dynamic changes in perinatal stages, suggesting DNA methylation is involved in nervous system differentiation processes [Ono, et al., 1993; Tawa, et al., 1990; Wilson, et al., 1987]. Furthermore, neural differentiation can be blocked by the demethylating agent 5-azacytidine in an *in vitro* model [Persengiev and Kilpatrick, 1996].

In clear contrast to the dependence of differentiated somatic cells on functional DNA methylation, undifferentiated embryonic stem (ES) cells can tolerate hypomethylation caused by inactivation of *Dnmt1* or both *Dnmt3a* and *Dnmt3b*. *Dnmt1* knockout ES cells, even though being strongly hypomethylated, are viable and grow without any limitations regarding their proliferative capacity if maintained in non-differentiation culture conditions [Lei, et al., 1996]. Similarly, combined deletion of *Dnmt3a* and *Dnmt3b* in ES cells did not have any effect on their viability and retained their stem cell characteristics. Even triple knockout ES cells, depleted of *Dnmt1*, *Dnmt3a* and *3b* and consequently devoid of any CpG methylation, maintained stem cell charac-

teristics, proliferation ability and normal chromosomal order [Tsumura, et al., 2006]. However, if any of the knockout ES cell lines was subjected to differentiation, either by using differentiation-driving culture conditions or by blastocyste injection, hypomethylated cells succumb to rapid cell death. This clear discrepancy between differentiated cells and undifferentiated ES cells, raised the question as to whether adult somatic tissue stem cells are critically dependent on DNA methylation like their differentiated counterparts, or can tolerate hypomethylation like ES cells.

This issue has been tackled in various studies applying several differentiation models. *Dnmt1*, which is highly expressed in the mammalian brain [Goto, et al., 1994], was specifically deleted in neural stem cells by combining *Dnmt1*<sup>lox</sup> mice with CamK-Cre transgenic mice, in which Cre expression is under the control of the neuronal camodulin-kinase II $\alpha$  (CamK). *Dnmt1* deficiency resulted in hypomethylation of central nervous system stem-/precursor cells after cell division causing functional impairment and cell death [Fan, et al., 2001]. A more recent study applied a well-defined *in vitro* differentiation system, which encompasses the differentiation from mouse ES cells via a committed progenitor stage to terminally differentiated glial cells [Bibel, et al., 2007]. DNA methylation analysis of promoter sequences of all three differentiation steps revealed a strong gain of CpG promoter methylation, underlining the importance of DNA methylation for somatic differentiation processes [Mohn, et al., 2008]. Furthermore, DNA methylation was shown to essentially control the regulation between self-renewal- and differentiation–programs in epidermal tissue. Knockdown of *Dnmt1* led to premature differentiation at the expense of self-renewal properties by lost repression of differentiation driving genes [Sen, et al., 2010]. Some of the most important scientific advances in the field of differentiation processes and stem cell biology have been made in studies of the hematopoietic system. The hematopoietic system itself and findings concerning genetic and epigenetic differentiation processes which have been gathered studying this system will be introduced in detail in the next chapter.

## 1.4 Regulation of hematopoietic differentiation

The hematopoietic system, encompassing the collectivity of blood cells and several other cell types, such as dendritic cells, neuron-attached microglia or bone resorbing osteoclasts, is a prototype experimental model system, which has been extensively

studied in the last decades. The importance of this model system is, at least in part, due to the many experimental advantages it offers. Hematopoietic cells are found in several organs, like bone marrow, spleen, lymph nodes, thymus, the peritoneal cavity and the peripheral blood, all of which can relatively easily be isolated, processed to single cell suspensions and used for experimental purposes. Development of fluorescence activated cell sorting (FACS) accompanied by the production of a constantly increasing number of fluorochrome-conjugated antibodies against cellular surface antigens, allowed a very precise characterization of hematopoietic cells. Additionally, this technique renders the possibility to separate distinct cell populations on the basis of their surface marker composition. Furthermore, differentiation processes of hematopoietic cells can be followed both *in vitro*, by providing specific differentiation conditions like cytokines or feeder cells, or *in vivo*, by transplantation of cells. Organ suspensions or sorted cell populations can be transferred by intravenous injection into recipient animals providing a precious tool to follow up cell fates within a chosen environment or experimental setting.

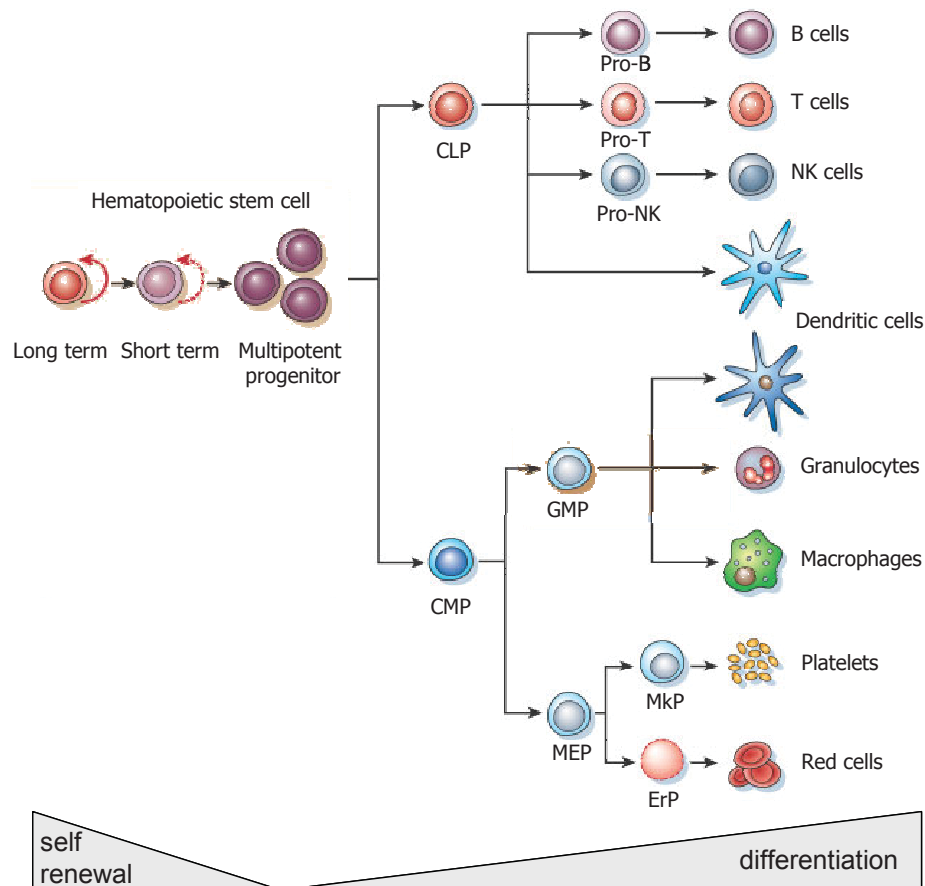
#### 1.4.1 The hematopoietic system

Hematopoiesis describes the formation of blood cells and several other specified cell types mentioned earlier, a process requiring constant replenishment due to high turnover rates of cells. An estimated number of one trillion blood cells is formed in humans every day for the whole life [Ogawa, 1993]. This remarkable cell renewal process depends on hematopoietic stem cells (HSCs). The original pool of HSCs is formed during embryogenesis in a complex developmental process that involves several anatomical sites. In the mouse embryo, hematopoiesis occurs by day 8 postconception in the yolk sac and the AGM (aorta-gonad-mesonephros region) followed by the fetal liver. At birth HSCs colonize the bone marrow where they reside in specialized niches, providing a lifelong resource for blood cell formation [Mikkola and Orkin, 2006]. HSCs display the apex of the differentiation hierarchy of the hematopoietic system (**Figure 5**). They are functionally defined at single-cell level by their ability to self-renew and to give rise to all lineages of blood cells [Becker, et al., 1963; Jordan and Lemischka, 1990; Lemischka, et al., 1986; McCulloch and Till, 1960]. These characteristics are founded in the HSCs' capability to undergo asymmetric cell divisions, meaning that an individual HSC can give rise to

functionally not-equivalent paired daughter cells [Ema, et al., 2000; Takano, et al., 2004]. Mouse transplantation experiments were applied to phenotypically identify the cell population which is equipped with these described characteristics, or, more practically, which is able to provide long-term reconstitution of a damaged bone marrow. These studies revealed that stem cell activity is harbored in a cell population described by the following cell surface marker combination: HSCs are negative for markers indicative of a specific lineage identity (so called lineage antigens; lin), and they are positive for both c-kit and sca-1 leading to their naming as LSK cells (lin<sup>-</sup>sca-1<sup>+</sup>c-kit<sup>+</sup>). Differences in long-term reconstitution potential led to sub-division of the LSK compartment into long-term HSCs (LSK, CD34<sup>-</sup>Flt3<sup>-</sup>), short-term HSCs (LSK, CD34<sup>+</sup>Flt3<sup>-</sup>) and multipotent progenitors (LSK, CD34<sup>+</sup>Flt3<sup>+</sup>) [Adolfsson, et al., 2001; Osawa, et al., 1996; Yang, et al., 2005]. The latter population was shown to have significantly lost megakaryocytic potential, but exhibiting bias for lymphoid differentiation leading to its alternative designation as lymphoid primed multipotent progenitor (LMPP) [Adolfsson, et al., 2005]

As described in **Figure 5**, hematopoietic differentiation consists of two major lineage pathways, the lymphoid and the myeloerythroid pathway. Differentiation via several lineage-specific precursor stages gives rise to a number of mature cell types, exerting a variety of essential physiological functions. Red blood cells and platelets are responsible for oxygen transport and blood clotting, respectively. All other cell types together build the immune system, with the myeloid macrophages and granulocytes composing the innate immune system, and lymphocytes, encompassing B cells, T cells and natural killer cells, forming the adaptive immune system.

Taken together, HSCs give rise to progeny that progressively lose multipotency and self renewal capacity and become restricted to one lineage [Metcalf, 1999]. The complexity of this system clearly calls for a tight regulation of cell fate choices in the course of hematopoietic differentiation. These regulatory networks have been subject to countless studies, revealing the involvement of genetic and epigenetic processes in the orchestration of cell fate decisions, both of which will be introduced in the following sections.



**Figure 5: Scheme of the hematopoietic system**

Hematopoietic stem cells (HSC) with self-renewal capacities (red arrows) can be subdivided into long-term and short-term HSC and multipotent progenitors (MPP). Differentiation follows two major pathways: the lymphoid branch starting with the common lymphoid progenitor (CLP) and the myeloerythroid branch developing from the common myeloid progenitor (CMP). Formation of mature blood cells is achieved via various lineage specific progenitor stages. NK, natural killer; GMP, granulocyte-macrophage precursor; MEP, megakaryocyte-erythrocyte precursor; MkP, megakaryocyte precursor; ErP, erythrocyte precursor. Adapted from [Reya, et al., 2001].

### 1.4.2 Genetic regulation of hematopoietic differentiation

A great number of transcription factors have been identified to be involved in the pathway choice of multipotential cells in the hematopoietic system. For example, C/EBP $\alpha$  (CCAAT/enhancer binding protein  $\alpha$ ) and PU.1 were shown to belong to a group of master switches instructing myeloid differentiation [Rosenbauer and Tenen, 2007]. Knockout mice for either factor display lack of distinct myeloid cell populations like granulocyte-macrophage progenitors (GMPs) and granulocytes (C/EBP $\alpha^{-/-}$ ) or mature myeloid cells (PU.1 $^{-/-}$ ) [Iwasaki, et al., 2005; Zhang, et al., 1997].

Furthermore, GATA-1, was shown to be indispensable for erythroid development as it functions as a major regulator on several erythroid-specific genes [Orkin, 1995; Simon, et al., 1992]. As to the lymphoid lineage, the factors EBF1, PAX5 and NOTCH-1 were proven to be essential for proper B-cell- and T-cell-development, respectively [Lin and Grosschedl, 1995; Nutt, et al., 1999; Radtke, et al., 1999].

Although differentiation appears to be a unidirectional process with subsequent steps of lineage commitment, experimental manipulation of transcription factor dosages have been shown to allow a cell to overcome a certain commitment and to chose an alternative fate. This was, for example, impressively shown by Thomas Graf's group, who managed to convert mature B cells into macrophages by forced expression of the transcription factor C/EBP $\alpha$ .

Furthermore, it has been widely demonstrated that dysregulation of transcription factor expression can dramatically interfere with physiological differentiation pathways and might lead to cancer formation. For example, reduced expression of PU.1 in HSCs and myeloid progenitors induced a differentiation block of the myeloid lineage leading to an aggressive form of acute myeloid leukemia (AML) in mice [Rosenbauer, et al., 2004]. On the other hand, enforced expression of NOTCH1 was shown to be a potent inducer of T-cell acute lymphoid leukemia (T-ALL) in mice and in fact over 50 % of human cases of T-ALL display activating mutations of NOTCH1 [Aster, et al., 2000; Ferrando, 2009].

Both, the fact that manipulated expression of transcription factors allows to overcome restricted cell fates in trans-differentiation processes, as well as the possibly fatal consequences of dys-regulation, underline the power of these factors. This power has to be very tightly controlled, which is achieved in a finely tuned network of activators and repressors orchestrated by means of epigenetics.

### **1.4.3 Epigenetic regulation of hematopoietic differentiation**

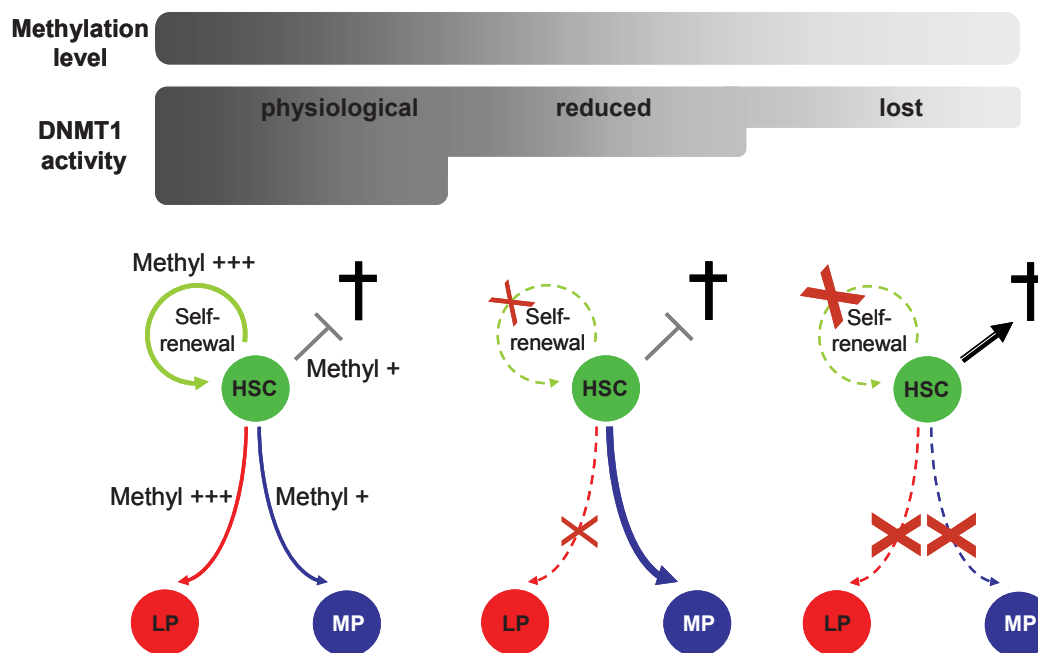
As this thesis deals with the epigenetic process of DNA methylation, this chapter will focus on the role of this specific process in hematopoietic differentiation. In 2007, DNMT3a and DNMT3b were reported to be essential for proper functioning of HSCs. Combined loss of both de novo methyltransferases in HSCs drastically impaired their self-renewal capacity leading to their inability of long-term reconstitution in transplantation assays. However, *Dnmt3a/Dnmt3b* deficient HSCs maintained normal

differentiation potential as both lymphoid and myeloid progeny were given rise to by these cells [Tadokoro, et al., 2007].

Last year, our group reported a pivotal role for *Dnmt1* in HSC fate choices [Broske, et al., 2009]. Conditional knockout of the *Dnmt1* gene induced apoptosis in HSCs causing rapid death of mice as a consequence of complete bone marrow failure. Due to this dramatic phenotype further studies applied a knockdown mouse model of *Dnmt1*, combining one *Dnmt1* knockout allele (*Dnmt1<sup>c</sup>*, further termed as *Dnmt1<sup>-</sup>*) with a hypomorphic allele. This allele, termed *Dnmt1<sup>chip</sup>* (chip = cDNA homologous insertion protocol) was created by a *Dnmt1* cDNA knockin into the *Dnmt1<sup>-</sup>* allele, resulting in functional *Dnmt1* expression at levels lower than those of the wild type [Tucker, et al., 1996]. *Dnmt1<sup>-chip</sup>* mice [Gaudet, et al., 2003] are viable but exhibit genomic hypomethylation due to reduced expression levels of *Dnmt1*. Analysis of the hematopoietic system revealed greatly diminished self-renewal capacities of *Dnmt1<sup>-chip</sup>* HSCs and a severe block in lymphoid differentiation, which was found to be caused by the *Dnmt1<sup>-chip</sup>* HSCs' inability to silence myeloerythroid genes through DNA methylation. As summarized in **Figure 6**, these results showed that distinct levels of DNA methylation are required to control different functional programs in HSCs, such as self-renewal and alternative lineage choices [Broske, et al., 2009].

These observations were supported by a simultaneous report from Stuart Orkin's lab, which stated a similar importance for DNMT1 in HSCs [Trowbridge, et al., 2009]. Moreover, a very recent publication offered a comprehensive map of DNA methylation in various differentiation stages of the hematopoietic hierarchy, which revealed that differentiation towards a lymphoid cell fate involves drastically higher-level methylation at differentially methylated regions compared to myeloid commitment [Ji, et al., 2010]. Although our knowledge about how epigenetic processes direct cell fates and which specific molecular interactions contribute to this are still in its infancy, DNA methylation is obviously crucially involved in the network of orchestration.





**Figure 6: Model of DNA methylation dosage effects on HSC multipotency**

Alternative HSC fates have different DNA methylation dosage requirements: In a physiological situation self-renewal and lymphoid differentiation rely on high methylation levels, whereas lower levels suffice to prevent apoptosis and allow myeloid differentiation. Reduced DNA methylation level (middle part), resembling the situation in *Dnmt1*<sup>-*chip*</sup> mice, still allows some essential hematopoiesis but greatly diminishes self-renewal capacity and lymphoid pathway choice. If, however, DNMT1 activity is completely lost (right panel), HSCs undergo apoptosis, excluding any other fate usually offered by the HSC's multipotency. MP, myeloid progeny; LP, lymphoid progeny; †, apoptosis; Methyl+++, high methylation level required; Methyl+, low methylation level required. Adapted from [Broske, et al., 2009]

With the role of DNA methylation being that critical for the normal functioning of an organism, it stands to reason that any defects in the methylation machinery might dramatically interfere with the physiological balance and therefore being a plausible cause for disease.

## 1.5 DNA methylation and disease

A growing number of human diseases is known to be caused by or at least associated with improperly established or maintained epigenetic information. Among them are several diseases categorized as imprinting disorders such as Beckwith-Widemann syndrome, Prader-Willi syndrome and Angelman syndrome [Robertson,

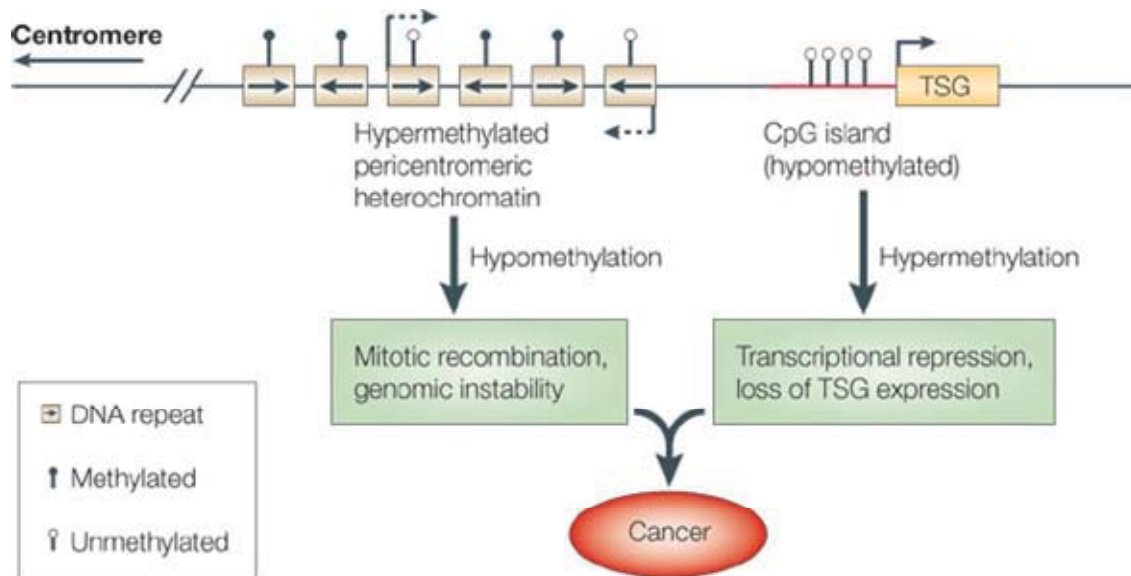


2005]. Mutations in the *DNMT3B* gene are cause for the majority of ICF (Immunodeficiency, centromeric instability and facial anomalies) syndrome cases. Hypomethylation due to partial loss of *DNMT3B* activity leads to instability of pericentromeric heterochromatin and disruption of gene expression patterns [Hansen, et al., 1999; Xu, et al., 1999]. Mutations in the *MECP2* gene coding for one of the 'readers' of DNA methylation information (see 1.2.2) are cause for the neuro-developmental disorder Rett syndrome [Amir, et al., 1999].

However, most important for this thesis is the fact that almost every cancer displays an altered DNA methylation pattern [Yoo and Jones, 2006], a connection which will be further introduced in the next chapter.

### 1.5.1 DNA methylation and cancer

A link between DNA methylation and cancer was first demonstrated in 1983, when it was shown that cancer cell genomes are hypomethylated relative to their normal counterparts [Feinberg and Vogelstein, 1983]. This reduction of methylation marks is primarily found in repetitive satellite sequences of the genome such as in pericentromeric heterochromatin. CpGs in such regions are usually methylated and hypomethylation as found in cancer is associated with genomic instability (**Figure 7**, left) [Robertson, 2005; Yoder, et al., 1997b]. For example several types of cancer, like Wilms tumor, breast and ovarian cancer, frequently contain chromosomal translocations with breakpoints in the pericentromeric DNA of chromosomes 1 and 16, which specifically correlate with hypomethylation in the respective regions [Qu, et al., 1999]. This suggests that demethylation of satellite sequences might predispose to their breakage and recombination [Feinberg and Tycko, 2004]. Furthermore, hypomethylation at specific loci might lead to activation of oncogenes. However, although known oncogenes like *c-myc* and *H-ras* were found to be hypomethylated in human tumors, there is no compelling evidence that such local hypomethylation causes overexpression of the respective genes to promote tumor development [Costello and Plass, 2001]. In fact, DNA hypomethylation in tumors has been associated with transcriptional activation of an unexpectedly low number of genes [De Smet and Lorient, 2010].



**Figure 7: Aberrant DNA methylation in cancer**

This scheme describes the nature of aberrant DNA methylation patterns found in tumor cells. Repeat-rich heterochromatic regions are frequently hypomethylated, contributing to genomic instability through increased mitotic recombination events. Hypermethylated CpG islands in tumor suppressor gene (TSG) promoters serves to transcriptionally silence growth-regulatory genes [Robertson, 2005].

Although most tumors exhibit a global reduction of 5-methylcytosine, the opposite phenomenon, DNA hypermethylation, is found at specific loci of tumor cells. Such aberrant hypermethylation usually occurs at CpG islands of tumor suppressor gene (TSG) promoters, most of which are unmethylated in normal somatic cells. Consequently, TSGs become transcriptionally silenced in cancer cells (**Figure 7** right) allowing the cell to discard cell progression antagonists. Aberrant silencing was for example shown for the cell cycle regulators  $p14^{ARF}$ ,  $p16^{INK4a}$  and the retinoblastoma gene ( $Rb$ ) [Esteller, et al., 2000b; Merlo, et al., 1995; Simpson, et al., 2000] as well as for the  $BRCA1$  gene involved in DNA repair [Esteller, et al., 2000a].

Several tumor-related observations have been made in studies with *Dnmt* mouse models. In 1995, Rudolf Jaenisch's group showed that hypomethylation, achieved by combination of a *Dnmt1* heterozygous mouse model with DNMT-inhibitor (5-azadeoxycytidine) treatment, suppressed the development of intestinal neoplasia in a tumor-prone  $APC^{Min}$  mouse background [Laird, et al., 1995]. Furthermore, overexpression of *Dnmt3b* in tumor-prone  $APC^{Min}$  mice was shown to promote tumorigenesis [Linhart, et al., 2007]

In contrast to this, other studies suggested a rather pro-oncogenic effect of DNA hypomethylation. Tumor-prone mice carrying heterozygous mutations in the tumor-suppressor genes *Nf1* (Neurofibromatosis 1) and *p53* were investigated for loss of heterozygosity (LOH) in either a *Dnmt1* hypomorphic or wildtype context revealing a significant increase in LOH rate in a hypomorphic context [Eden, et al., 2003]. In addition, some hypomorphic *Dnmt1*<sup>-*chip*</sup> mice develop T-cell lymphomas later in life [Gaudet, et al., 2003].

This ambivalent role of DNA methylation in tumorigenesis was most impressively demonstrated by Yamada and colleagues who demonstrated opposing effects of DNA hypomethylation on intestinal and liver carcinogenesis. Whereas hypomethylation promoted the development of early lesions in colon and liver through LOH in a *Dnmt1*<sup>-*chip*</sup>, *APC*<sup>*Min*</sup> mouse model, later stages of intestinal tumorigenesis were suppressed in these mice [Yamada, et al., 2005].

Taken together, these data suggest a crucial role for DNA methylation in cancer initiation and progression, the precise functioning and mechanisms, however, are not fully understood yet. Howsoever unclear the role of DNA methylation in tumorigenesis is, it should be mentioned that *Dnmts* themselves are frequently overexpressed in tumors. This has, for example, been shown for *Dnmt1* in colon cancer [Lee, et al., 1996] and leukemia [Melki, et al., 1998] as well as for *Dnmt3a* and *3b* in cases of acute myeloid leukemia [Mizuno, et al., 2001]. A very recent genome-wide study of AML patient samples revealed highly recurrent mutations of the *Dnmt3a* gene. Even though these mutations were not accompanied by lower levels of 5-methylcytosine and no methylation dependent changes in gene expression were observed, patients with mutated *Dnmt3a* showed significantly reduced overall survival. Despite these strong correlations, the mechanism by which these mutations act are yet unknown [Ley, et al., 2010].

### 1.5.2 Epigenetic therapy of cancer

The potential reversibility of DNA methylation marks suggests that they are a viable target for a cancer treatment aiming to restore a more normal DNA methylation pattern. Treatment of tumor cells with demethylating agents intends to revert hypermethylation-induced gene silencing. Targeting of DNMT activity, however, is unspecific and cannot be applied for direct demethylation at specific loci. Moreover,

demethylating therapy bears the risk of aggravating the situation by further demethylating an already hypomethylated genome or activating potentially deleterious genes, such as oncogenes [Yoo and Jones, 2006]. Nevertheless, DNA methylation inhibitors have been successfully approved for the treatment of specific tumor types, primarily hematological malignancies, or are currently tested in clinical trials. Most promising results have been obtained with nucleoside-analogue inhibitors such as 5-Azacytidine, 5-Aza-2'deoxyctidine (5-Aza-dC; also decitabine) and Zebularine [Yoo and Jones, 2006].

Tumor suppressor gene reactivation was directly shown for patients suffering from myelodysplastic syndrome (MDS), who were treated with decitabine. Comparing patient samples before and after treatment revealed reversal of hypermethylation of the *p15* gene, a negative cell cycle regulator, accompanied by its enhanced expression [Daskalakis, et al., 2002].

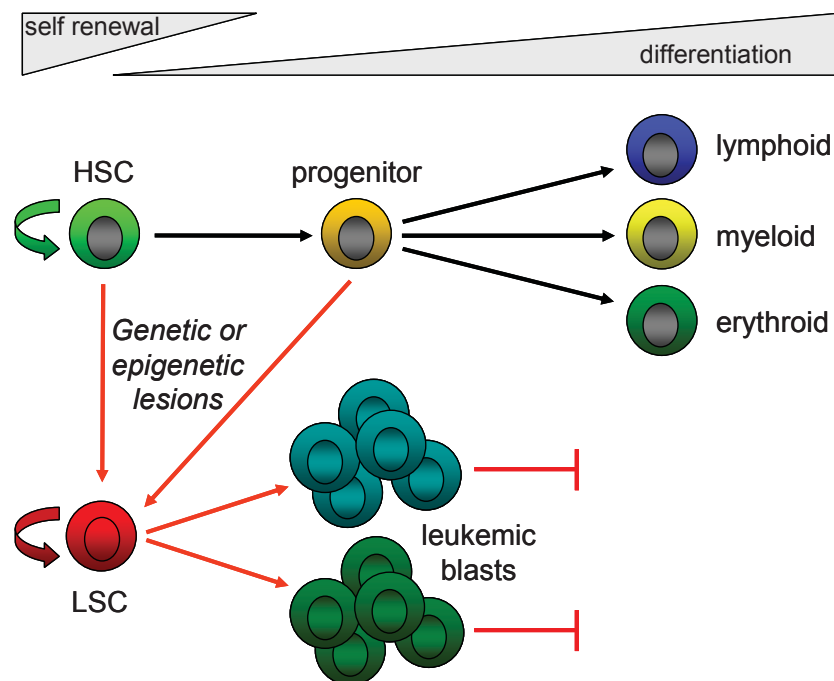
As hypermethylation is not the only epigenetic mechanism which silences TSG expression, but usually occurs in combination with repressive histone modifications, such as histone deacetylation, the effectiveness of demethylating drugs alone might be limited. Therefore, a combinatorial treatment with demethylating agents and histone deacetylase (HDAC) inhibitors, such as trichostatin A (TSA), might display an improved therapeutic option. Combined treatment of a colorectal carcinoma cell line with 5-Aza-dC and TSA resulted in synergistic reactivation of hypermethylated TSGs, whereas neither agent alone was able to do so [Cameron, et al., 1999]. Given this synergy of epigenetic therapeutics, combinations of DNMT and HDAC inhibitors are currently tested in clinical trials for treatment of acute myeloid leukemia (AML) and MDS [Chen, et al., 2010].

Given the pivotal role of DNA methylation in malignancies, drugs targeting methylation aberrations might display a powerful therapeutic approach. If, however, the underlying molecular mechanisms were better understood and the target cells more clearly identified, the power of such therapeutics could be by far better exploited.

### **1.5.3 Leukemia stem cells**

The cancer stem cell concept describes a hierarchical model of a tumor cell population with a cancer stem cell (CSC) at the apex, analogous to a tissue stem cell

at the apex of a given somatic differentiation system. In 1997, the first cancer stem cell was identified in human AML as a small subpopulation which was able to transfer the disease from the human patient to an immunodeficient mouse [Bonnet and Dick, 1997]. These cells were designated as leukemia initiating cells or leukemia stem cells (LSC). The LSC (as all CSCs) shares functions with its normal, non-malignant counterpart, the HSC, such as unlimited self-renewal and the ability to give rise to more mature progeny (**Figure 8**). These abilities are maintained or reacquired by epigenetic or genetic aberrations creating a cell with infinite proliferation potential, which gives rise to phenotypically diverse progeny [Passegue, et al., 2003]. The progeny, also called the leukemic blasts, are usually arrested at a not terminally differentiated stage and account for the major mass of tumor cells.



**Figure 8: The leukemia stem cell concept**

This simplified scheme depicts the hierarchical organization of both normal hematopoiesis and leukemia. Leukemia stem cells (LSCs) are formed by genetic or epigenetic events in HSCs or progenitors. LSCs give rise to leukemic blasts which follow a certain lineage pathway but are not able to terminally differentiate.

Concluding, LSCs have to be (re-)equipped with stem cell properties in the process of their formation, however, which genetic and epigenetic events underlie this transformation remains poorly understood both in case of leukemia as well as for

other cancers.

The existence of CSCs is of great clinical relevance, as their unique stem cell properties might enable them to escape conventional cancer therapy which is designed to target the rapidly cycling and highly proliferating cancer blasts. This inability to eradicate CSCs might be responsible for the disease relapses of cancer patients, as the CSC is able to replenish the tumor cell population even after the majority of blasts has been eradicated. Consequently, specific anti-CSC therapy is of immense clinical importance, however, the restricted knowledge about the molecular characteristics of these cells has not allowed the design of such a therapy so far.

## **1.6 Aim of this thesis**

Comprehension of the genetic and epigenetic basis of leukemia stem cell (LSC) formation is an essential prerequisite for the design of tailored anti-LSC therapy. However, the precise mechanisms underlying this process are only insufficiently understood. Aberrant patterns of DNA methylation, one of the major epigenetic processes, are a hallmark of leukemia, as it is for all cancers. Due to the reversibility of epigenetic alterations compared to genetic mutations, therapeutics targeting epigenetic processes might be of great potential for such applications. DNA methylation was shown to be crucially involved in hematopoietic stem cell (HSC) functioning, both in respect to self-renewal as well as differentiation fates. Given the similarities of HSCs and LSCs, one might hypothesize that DNA methylation plays a comparably important role in LSCs. This thesis aims to investigate how DNA methylation is involved in self-renewal and differentiation choices of LSCs and which impact it has on the (re-)establishment of stemness in LSC formation.

## 2 Materials and Methods

### 2.1 Materials

#### 2.1.1 General equipment

7300 Real Time PCR System	Applied Biosystems
Agarose gel chambers	Biosteps
Centrifuge Pico 17	Heraeus
F96 MicroWell™ Plates	Thermo Scientific
FACS Calibur	BD
FACS LSRII	BD
FACS Fortessa	BD
FACS Aria I	BD
FACS Aria II	BD
FACS Aria III	BD
Geldoc 2000	Biorad
Hybridization oven Hybridiser HB-1D	Techne
Incubator Steri Cult 200	Forma Scientific
Incubator	Binder
Laminar Flow Hood	BDK
Luminometer Centro 960	Berthold Technologies
Mastercycler Gradient	Eppendorf
Microscope DMIL	Leica
Multicentrifuge 3 S-R	Heraeus
Nanodrop Spectrophotometer	PeqLab
Nytran SuperCharge Southern/Northernblot membrane	Schleicher&Schuell
Power supply EV231	Consort
Stereomicroscope MZ16 with halogen cold light source KL1500 LCD	Leica
Stratalinker 2400	Stratagene
SDS gel chambers	Biorad
Thermomixer compact	Eppendorf
Vortex Genie 2	Scientific Industries
XAR film	Kodak

### 2.1.2 Cell culture equipment

Cell culture dishes, sterile	BD
Cell strainer, sterile, 40, 70 and 100 $\mu$ m	BD
Centrifuge tubes, sterile, different sizes	TPP or Falcon
Cryotubes, sterile, 1.2 ml	Nunc
Disposable scalpel for single-use, steril	Cutfix
Hemocytometer (Neubauer cell counter chamber)	Superior Marienfeld
MACS® Cell Separation LS Columns	Miltenyi
MACS® MultiStand	Miltenyi
MidiMACS™ Separator	Miltenyi
Needles for single-use, sterile, different sizes	Braun
Polystyrene tubes, 5 ml	BD Falcon
Rotilabo Filter sterile, 0.22 and 0.45 $\mu$ m PVDF	Roth
Serological pipettes	Falcon
Syringes for single-use, sterile, different sizes	Braun, Omnifix, BS Plastic

### 2.1.3 Mouse dissection equipment

Dissecting board and pins	
EDTA-treated canula	Brand
Forcipes and scissors of different sizes	
Scalpels, sterile	Cutfix

### 2.1.4 Chemicals and reagents

4',6-Diamidino-2-phenylindol (DAPI)	Sigma Aldrich
5-aza-2'-deoxycytidine	Sigma-Aldrich
$\alpha$ 32-PdCTP	Amersham
Agarose	Roth
Annexin binding buffer	(BD
$\beta$ -Mercaptoethanol	Roth
Bromphenol blue	Roth
Bovine serum albumin (BSA)	Roth
Chloroform/Isoamylalcohol	Roth
dNTPs	Fermentas



DTT	Roth
Ethanol absolute	
Ethidium bromide	Roth
Ethylenediaminetetraacetate (EDTA)	Roth
Formaldehyde	Roth
Giemsa Stain	Fluka
Glacial acetic acid	Roth
Glucose-1	Roth
Glycerol	Roth
Hepes	PAA
High molecular weight marker	Fermentas
Histofix	Roth
Hybri-Quick	Roth
Isoamyl alcohol	Roth
Isopropyl alcohol	Roth
KCl	Roth
KH <sub>2</sub> PO <sub>4</sub>	Roth
LB Agar	Roth
Low molecular weight marker	Fermentas
May-Gruenwald Stain	Fluka
MgCl <sub>2</sub>	Roth
Methanol	Roth
Na <sub>2</sub> HPO <sub>4</sub>	Roth
NaCl	Roth
Phenol	Proteinase K (Invitrogen)
Poly(I:C)	GE-Healthcare or Invivogen
Propidium iodide	Sigma Aldrich
RNase free water	Quiagen
S-Adenosylmethionine (SAM)	New England Biolabs
Sodium dodecyl sulfate (SDS)	Roth
Trichlormethan (Chloroform)	Roth
Tris base	Roth
Tris-Cl	Roth
Trizol: peqGOLD® TriFast	PeqLab
Trypan blue solution	Sigma

Xylene cyanol

Roth

### 2.1.5 Buffers and solutions

6x Loading buffer for agarose gel electrophoresis

0.25 % Bromophenol blue  
0.26 % Xylene cyanol  
30 % Glycerol

20x SSC

3 M NaCl  
0.3 M sodium citrate  
1 mM EDTA

ACK red blood cells lysis buffer

0.15 M  $\text{NH}_4\text{Cl}$   
10 mM  $\text{KHCO}_3$   
0.1 mM EDTA, pH 7.3

FACS buffer

2 % FCS  
2 mM EDTA  
in PBS

Freezing medium

10 % DMSO  
in FCS

Phosphate buffered saline (PBS)

137 mM NaCl,  
2.7 mM KCl,  
10 mM  $\text{Na}_2\text{HPO}_4$ ,  
2 mM  $\text{KH}_2\text{PO}_4$ 

Southern blot wash solution I

100 ml 20 x SSC  
10 ml 20 % SDS  
890 ml water

Southern blot wash solution II

10 ml 20 x SSC  
10 ml 20 % SDS in  
980 ml water

Tail digestion buffer

10 mM Tris-Cl pH 8.0  
10 mM EDTA pH 8.0  
50 mM NaCl  
0.5 % SDS

TE buffer

10 mM Tris  
1 mM EDTA, pH 7.5

Tris-acetate-EDTA buffer (TAE)

242 g Tris base  
57.1 ml Glacial acetic acid  
100 ml 0.5 M EDTA, pH 8.0  
in 20l water

### 2.1.6 Cell culture media and reagents

Bovine serum albumin

Roth

Dulbecco's modified Eagle's Medium (DMEM), high  
Glucose (4.5 g/l)

PAA

Dulbecco's Phosphate Buffered Saline 1 x, without Ca  
& Mg

PAA

Fetal Calf Serum (FCS)	Biochrom
Fungizone Antimycotic agent	Invitrogen
Gentamicin	PAA
Iscove's modified DMEM (IMDM)	PAA
MEM Alpha Modification, with L-Glutamine, without Ribonucleosides	PAA
MethoCult® M3234	Stem Cell Technologies Inc.
N,N-dimethylsulfoxide (DMSO)	Roth
Penicillin/Streptomycin, 100 x Concentrate	Roth
Polybrene (Hexadimethrine bromide)	Sigma-Aldrich
Retronectin	Takara
RPMI 1640 cell culture medium	PAA
Stable Glutamine, 200 mM Concentrate	PAA
Trypsin EDTA (1:250) 1 x Concentrate	PAA

### 2.1.7 Enzymes and appending buffers

Collagenase	Sigma
Proteinase K	Invitrogen
RNase out	Fermentas
DNaseI	Fermentas
10 x Dnase I buffer	Fermentas
EcoR I	Fermentas
10 x Buffer for EcoR I	Fermentas
HindIII	Fermentas
HpaII	Fermentas
MspI	Fermentas
NcoI	Fermentas
10 x Tango buffer	Fermentas
T4 Ligase	New England Biolabs
10 x T4 Ligase buffer	New England Biolabs
Taq polymerase	Fermentas
KCl buffer for PCR	Fermentas
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> buffer for PCR	Fermentas
Superscript II	Fermentas

5 x First round buffer	Fermentas
SssI: CpG Methyltransferase	New England Biolabs
10 x NEBuffer 2	New England Biolabs
XhoI	Fermentas
10 x Buffer R	Fermentas

### 2.1.8 Kits

Calcium Phosphate Transfection Kit for transient transfection of PLAT-E cells	Invitrogen
Dual-Luciferase <sup>®</sup> Reporter Assay System	Promega
Invisorb Spin DNA extraction kit for gel extraction	Invitex
Genomic DNA Invisorb Kit III for DNA isolation	Invitex
Rapace Kit for isolation of DNA fragments	Invitex
Rediprime II DNA Labeling System for radioactive labeling of DNA	Amersham
RNeasy Micro Kit for RNA isolation	Qiagen
RNeasy Mini Kit for RNA isolation	Qiagen

### 2.1.9 Antibodies

<b>Target Surface Receptor</b>	<b>Clone</b>	<b>Conjugates</b>	<b>Supplier</b>
B220/CD45R	RA3-6B2	PE/Cy5, APC, PE	Invitrogen or Biolegend
CD3ε	145-2C11	PE/Cy5, APC	BD Bioscience
CD4	GK1.5	PE/Cy5	BD Bioscience or Biolegend
CD48	HM48-1	APC	Biolegend
CD8α	53-6.7	PE/Cy5	Invitrogen or Biolegend
CD11b/ Mac1	M1/70	PE, APC	BD Bioscience or Biolegend
CD19	1D3	PE/Cy5, PE	Invitrogen or Biolegend
CD34	RAM34	FITC, biotin	eBioscience
CD45	30-F11	PE	BD Bioscience
CD45.1	A40	PE, APC	BD Bioscience or eBioscience
CD45.2	104	FITC, APC, biotin	BD Bioscience or

<b>Target Surface Receptor</b>	<b>Clone</b>	<b>Conjugates</b>	<b>Supplier</b>
			eBioscience
CD48	HM48-1	APC	Biolegend
c-Kit/ CD117	2B8	APC, AlexaFluor780	eBioscience or Biolegend
FcγRII/III	2.4G2	PE	BD Bioscience
Gr1/ Ly6C/G	RB6-8C5	PE/Cy5	Biolegend
Human CD4 (hCD4)	MEM-241	PE	Abcam
IgM	R6-60.2	APC	BD Bioscience
Sca1/ Ly6A/E	E13-161-7 or D7	FITC, PE/Cy7	eBioscience or BD Bioscience
Ter119	TER-119	PE/Cy5	Biolegend
Annexin-V			BD Bioscience
Streptavidin		APC/Cy7, PE/Cy7	BD Bioscience

### 2.1.10 Micro Beads

CD117 microbeads	Miltenyi
Compensation Beads Rat IgG, κ	BD Bioscience
Dynabeads® Sheep anti-Rat	Invitrogen

### 2.1.11 Cell lines

416B	[Dexter, et al., 1979]
E86	[Markowitz, et al., 1988]
K562	[Lozzio and Lozzio, 1975]
PLAT-E	[Morita, et al., 2000]
Pu1null	[Walsh, et al., 2002]

### 2.1.12 Cytokines

mFlt3L	Tebu Bio
mGM-CSF	Tebu Bio
mIL-3	Tebu Bio
mIL-6	Tebu Bio

mIL-7	Tebu Bio
mLif	R&D Systems
mSCF	Tebu Bio
mTPO	Tebu Bio

### 2.1.13 Mouse strains

<i>Dnmt1</i> <sup>-chip</sup> mice	[Gaudet, et al., 2003]
<i>Dnmt1</i> <sup>lox/lox</sup> mice	[Jackson-Grusby, et al., 2001]
Mx1Cre transgenic mice	[Kuhn, et al., 1995]
B6.SJL-Ptprca wild-type mice	Taconic
129ola wild-type mice	breeding in the mouse facility of the Max-Delbrück-Center, Berlin
129ola/B6.SJL F1 generation mice	breeding in the mouse facility of the Max-Delbrück-Center, Berlin

### 2.1.14 Oligonucleotides and gene expression assays

<i>Gene/ Oligo name</i>	<i>Application</i>	<i>Sequence (5' – 3')/ probe ID</i>
β.actin	TaqMan® Assay probe, endogenous control	NM_007393.1
β-actin fw	Expression PCR	AAG GAG ATT ACT GCT CTG GCT CCT A
β-actin rev	Expression PCR	ACT CAT CGT ACT CCT GCT TGC TGA T
CHIP242R	Genotyping PCR	CTG GTA GCC ACG GAA CTA GG
CHIP48F	Genotyping PCR	CTT GGA GAA CGG AAC ACA CA
Csf2ra fw	Expression PCR	CCA CGG AGG TCA CAA GGT CA
Csf2ra rev	Expression PCR	ACT CGC ACG TCG TCG GAC AC
D beta 2.1 extern	TCR rearrangement PCR	TAG GCA CCT GTG GGG AAG AAA C
J beta 2.7 extern	TCR rearrangement PCR	TGA GAG CTG TCT CCT ACT ATC
D beta 2.1 intern	TCR rearrangement PCR	GTA TCA CGA TGT AAC ATT GTG
J beta 2.7 intern	TCR rearrangement PCR	GGA AGC GAG AGA TGT GAA TC
DJ fw1	BCR rearrangement PCR	ACG TCG ACT TTT GTS AAG GGA TCT ACT ACT GT
DJ fw2	BCR rearrangement PCR	ACG TCG ACG CGG ASS ACC ACA GTG CAA CTG
DJ rev	BCR rearrangement PCR	GGG TCT AGA CTC TCA GCC GGC TCC

<b>Gene/ Oligo name</b>	<b>Application</b>	<b>Sequence (5' – 3')/ probe ID</b>
		CTC AGG G
Dnmt1	TaqMan® Assay probe	Mm00599783_g1
Dnmt1 ex32F	Genotyping PCR	ATC TCG GAA ACG CTG TGG GG
Dnmt1 ex32R	Genotyping PCR	CTT ACC TGA GGA AGG AGA CC
Dnmt1 lox 1	Excision PCR/ Genotyping PCR	GGG CCA GTT GTG TGA CTT GG
Dnmt1lox_gtyp2	Genotyping PCR	CCT GGG CCT GGA TCT TGG GGA
Dnmt1 lox 2	Excision PCR	TGA ACC TCT TCG AGG GAC C
Dnmt1 lox 3	Excision PCR	ATG CAT AGG AAC AGA TGT GTG C
Ebf1 fw	Expression PCR	CAA GAC AAG AAC CCT GAA ATG
Ebf1 rev	Expression PCR	GTA ACC TCT GGA AGC CGT AGT
Gapdh-206bp fw	Expression PCR	AAG GGC TCA TGA CCA CAG TC
Gapdh-206bp rev	Expression PCR	CAC ATT GGG GGT AGG AAC AC
Gata1	TaqMan® Assay probe	Mm00484678_m1
<i>Gata1</i> upstream fw Hind III	Gata1 promoter amplification	CCC AAG CTT TCC ACT CTG GGT GTC ACC TC
<i>Gata1</i> promoter rev Nco I	Gata1 promoter amplification	CAT GCC ATG GAT GCA GAG GAT TCA GCC ACC
IRES rev	Expression PCR	TCC AGA GGA ACT GCT TCC TT
Koprimer PGK	Genotyping PCR	GGG AAC TTC CTG ACT AGG GG
Mpo fw	Expression PCR	ATG CAG TGG GGA CAG TTT CTG
Mpo rev	Expression PCR	GTC GTT GTA GGA TCG GTA CTG
Mx1Cre fw	Genotyping PCR	CAA TTT ACT GAC CGT ACA C
Mx1Cre rev	Genotyping PCR	TAA TCG CCA TCT TCC AGC AG
Myc fw	Expression PCR	CTC TGA AAA GGA CTT ATT GAG GAA AC
Pax5 fw	Expression PCR	CGG GTC AGC CAT GGT TGT G
Pax5 rev	Expression PCR	GTG CTG TCT CTC AAA CAC G
Rag1 fw	Expression PCR	TGC AGA CAT TCT AGC ACT CTG GCC
Rag1 rev	Expression PCR	ACA TCT GCC TTC ACG TCG ATC CGG
Rag2 fw	Expression PCR	CAC ATC CAC AAG CAG GAA GTA CAC
Rag2 rev	Expression PCR	TCC CTC GAC TAT ACA CCA CGT CAA

Oligonucleotides were provided by Biotez or Metabion.

### 2.1.15 Vectors

MSCV-IRES-GFP (MIG)	
MSCV-Gata1-IRES-GFP	Murine <i>Gata1</i> cDNA was cloned into MIG via XhoI
MSCV-MLL-AF9-IRES-GFP	[Somervaille and Cleary, 2006]
MSCV-c-Myc-IRES-Bcl2	[Luo, et al., 2005]
pEYZ-Notch1-IC	Retroviral Notch1-IC expression construct
pCpGL-basic	[Klug and Rehli, 2006]
pCpGL- <i>Gata1</i> promoter	Murine <i>Gata1</i> promoter cloned into pCpGL basic via HindII and NcoI
pRL-null ( <i>Renilla</i> luciferase control vector)	Promega

Constructs were kindly provided by T. Somervaille (Stanford School of Medicine, Palo Alto, CA, USA), M.H. Tomasson (Department of Medicine and Genetics, Saint Louis, MO, USA), M. Rehli (University of Regensburg).

### 2.1.16 Software

<b>Software</b>	<b>Application</b>	<b>Supplier</b>
7300 System SDS Software	Real time RT-PCR analysis	Applied Biosystems
CellQuest Pro	Flow cytometry analysis	BD
FACSDiva	Flow cytometry analysis	BD
FlowJo	Flow cytometry analysis	Treestar
L-Calcul	Limiting dilution analysis	Stem Cell Technologies
TreeView (EisenSoftware)	MassARRAY data visualization	EisenSoftware

## 2.2 Methods

### 2.2.1 Molecular biology

#### 2.2.1.1 Preparation of genomic DNA

Genomic DNA extraction was performed following standard protocols. In brief, cells or tissues were digested with tail digest buffer supplemented with 10 µg/ml proteinase K at 56°C over night. Genomic DNA was purified with addition of a



phenol/chloroform/Isoamyl alcohol mixture and subsequent centrifugation (13000 rpm, 5 minutes, 4°C). The upper aqueous phase, which contained the DNA, was transferred into a new tube and precipitated with 1 ml pre-cooled ethanol or isopropyl alcohol. After centrifugation (13000 g, 20 minutes, 4°C) supernatant was removed and the DNA pellet was washed with 1 ml 70 % ethanol. After centrifugation (13000 g, 5 minutes, 4°C) supernatant was aspirated completely and the DNA pellet was air-dried until ethanol was completely evaporated. DNA was dissolved in H<sub>2</sub>O.

### 2.2.1.2 Extraction of RNA

For RNA extraction of cell-cultured cells or directly FACS-sorted cells, cells were resuspended in peqGOLD® TriFast and homogenized through vortexing (for cell numbers up to  $5 \times 10^5$ ) or through passing through a 16 gauge needle several times. After cell lysis, the samples were incubated at RT for 5 minutes to allow the dissociation of nucleoprotein complexes. 200 µl of Chloroform were added per ml of TriFast reagent, shaken vigorously and incubated for 3 minutes at RT. Phase separation was achieved by centrifugation (13000 g, 5 minutes, 4°C), and the upper, colorless aqueous phase, which contains the RNA, was transferred to a new tube. To precipitate RNA, 500 µl isopropyl alcohol per ml TriFast reagent was added to the samples, incubated for 10 minutes on ice and centrifuged (13000 g, 10 minutes, 4°C). Supernatant was removed and pellet was washed twice with 1 ml 75 % ethanol. After the final centrifugation step (13000 g, 10 minutes, 4°C) the supernatant was discarded and the pellet was air-dried until ethanol was completely evaporated. RNA was re-dissolved in RNase free H<sub>2</sub>O and incubated (10 minutes, 55°C). In general, RNA was stored at -80°C. All buffers, solutions, tips and other equipment were RNase free.

Alternatively, RNA was extracted using RNAeasy Kit according to the manufacturer's instructions.

### 2.2.1.3 Reverse transcription of RNA (cDNA synthesis)

To remove residual amounts of DNA in the RNA preparations, samples were treated with DNaseI: 5.2 µl RNA were incubated with 1 µl DNaseI, 0.8 µl 10 x DNaseI buffer and 1 µl RNase out (15 minutes, RT) followed by inactivation of DNaseI with 1 µl

EDTA (25  $\mu$ M) and incubation at 65°C for 10 minutes. 2  $\mu$ l dNTPs (10 mM) and 1  $\mu$ l random hexameres (300 nM) were added to the RNA and incubated for 5 minutes at 65°C to destroy secondary RNA structures. After a quick chill on ice, 4  $\mu$ l 5 x first strand buffer, 2  $\mu$ l DTT(mM) and 1  $\mu$ l RNase out were added and the mixture was incubated (10 minutes, 25°C). cDNA synthesis was started with adding 1  $\mu$ l Sperscript II and incubation (42°C, 50 minutes). The synthesis was stopped by incubation of the samples at 70°C for 15 minutes. cDNA was stored at -20°C. As a control, no RT samples (RNA samples which undergone DNase treatment but no reverse transcription) were generated exemplarily and used as a negative control in real time RT PCR analysis.

### 2.2.1.4 Agarose gel electrophoresis

Depending on the size of the DNA fragments to be analyzed, gels with an agarose content of 0.7-2% (w/v) were boiled in 1xTAE in a microwave. Once dissolved to a crystal clear solution, the agarose was cooled down to approximately 50°C. Ethidium bromide (0.5 $\mu$ g/ml) was added and poured into casted gel chambers. Gels were run in 1xTAE, at 100V. To determine DNA length of the fragments, an appropriate standard marker was used. DNA was loaded by addition of appropriate amounts of loading buffer and visualized under UV-light. For cloning purposes, DNA fragments of interest were excised with a scalpel and extracted using the Invisorb Spin DNA extraction kit (Invitex) following manufacturer's specifications.

### 2.2.1.5 Polymerase chain reaction (PCR) and quantitative (real time) RT PCR

PCR was carried out following standard procedures. All PCR reactions were carried out with the following reagents using the indicated final concentrations: 0.25  $\mu$ M forward primer, 0.25  $\mu$ M reverse primer, 0.5 mM dNTPs, 2 mM MgCl<sub>2</sub>, 1 x (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> or KCl buffer and 5 U/ $\mu$ l Taq polymerase. Either genomic DNA or cDNA was used as template. Amplified PCR products were separated and visualized on 1-2 % agarose gels containing 1  $\mu$ g/ml ethidium bromide. For quantitative PCR oligonucleotides were designed exon/exon spanning to reduce false positive signals from residual DNA contaminations. SYBR green real-time RT-PCR was performed using a 2 x SYBR green master mix whereas 2 x Taqman mix and 20 x Taqman assays were

used according instructions for TaqMan® Gene Expression Assays. 7300 System SDS Software was used to analyze results.

#### 2.2.1.6 *B- and T-cell receptor rearrangement PCR*

D-J rearrangements in the heavy Chain of Ig locus were detected by a PCR strategy employing two upstream degenerate primers binding 50 of the DFL/DSP element or the DQ52 element. The reverse primer was complementary to a binding site downstream of the JH4 segment. All three primers were used in a single PCR reaction in a multiplex PCR and the following reaction used in germline configuration, the DQ52 and JH4A primers will amplify the 2.15-kb germline fragment. D-JH1, D-JH2, D-JH3, and D-JH4 rearrangements involving either DFL, DSP, or DQ52 elements will be detected by the emergence of bands of ~1.46, ~1.15, ~0.73, and ~0.20 kb, respectively. The amplification protocol was an initial denaturation at 94°C for 1 minute followed by 35 cycles of 1 minute at 94°C, 1 minute at 60°C, and 1 minute 45 seconds at 72°C. Final extension was carried out at 72°C for 10 minutes.

The status of T-cell receptor (TCR) rearrangement was assessed as described previously [Iwasaki-Arai, et al., 2003]. Briefly, a nested PCR was applied to investigate the status of the TCR $\beta$  gene rearrangement. In the first PCR, the oligonucleotides J $\beta$ 2.1 ext and J $\beta$ 2.7 ext were used to amplify this part of the TCR $\beta$  gene from genomic DNA of FACS sorted myc/bcl2 leukemia cells. After 35 cycles of PCR (annealing temperature of 58°C) 20 % of the PCR product were used as template DNA for the second PCR, which was performed with the primer pair J $\beta$ 2.1 int and J $\beta$ 2.7 int. Amplified DNA of the second PCR was electrophoresed on a 1.2 % agarose gel.

#### 2.2.1.7 *Retroviral insertion analysis by Southern blot*

To analyze retroviral insertion sites genomic DNA was extracted from different MLL-AF9 leukemia samples and digested overnight with EcoRI, which cuts once within the viral sequence but not within the sequence encoding GFP. Digested DNA was electrophoresed on a 0.7 % agarose gel and subsequently capillary-blotted onto a nylon membrane. The blotted DNA was cross-linked to the membrane by UV light using a quantitative cross-linker. A 1 kb GFP-specific probe was released from a MIG vector

by cutting the plasmid with NcoI and HindIII and prepared by random-labeling with [ $\alpha$ - $^{32}$ P]dCTP using the Rediprime Labeling Kit. Hybridization of the nylon membrane with the  $P^{32}$  labeled GFP probe was carried out for 8-12 h in Roti-Hybri-Quick at 60°C and filters were washed twice in 2 x SSC, 0.1 % SDS and twice in 1 x SSC, 1 % SDS at 60°C for 15 minutes each. Washed filters were exposed to Kodak XAR film.

### 2.2.1.8 *In vitro* methylation

A ~800 bp long fragment of the murine Gata1 promoter was amplified by PCR using the oligonucleotides 'Gata1 promoter fw Hind III' and 'Gata1 promoter rev NcoI' and cloned into the CpG-free luciferase vector pCpGL-basic [Klug and Rehli, 2006] via HindIII and NcoI. *In vitro* methylation was performed as described in [Klug and Rehli, 2006]. 30  $\mu$ g of vector DNA was incubated with SssI (2.5 U/ $\mu$ g DNA) in the presence of 160  $\mu$ M S-Adenosylmethionine (SAM) for four hours at 37°C. Additional 160  $\mu$ M of SAM were added after two hours of incubation. The un-methylated control vector DNA was treated the same way but without the addition of SssI. After *in vitro* methylation, the plasmid DNA was purified with phenol/chloroform/isoamyl alcohol followed by isopropyl alcohol precipitation. Concentration of the purified DNA was assessed using the Nanodrop Spectrophotometer and subsequently used for luciferase assays (2.2.1.9).

Successful *in vitro* methylation was ensured by processing a CpG site containing control plasmid, the MSCV-IRES-GFP, in parallel. This vector was digested either with the methylation sensitive restriction endonuclease HpaII or the methylation insensitive MspI. The restriction pattern was analyzed by gel-electrophoresis.

### 2.2.1.9 Luciferase assay

K562 cells were transfected with luciferase vectors by electroporation as described previously [Nicolis, et al., 1989]:  $2 \times 10^7$  K562 cells were resuspended in 800  $\mu$ l of PBS and electroporated with 10  $\mu$ g methylated or un-methylated pCpGL-Gata1 promoter constructs and 1  $\mu$ g of *Renilla* luciferase at 400 V with a capacitance of 400  $\mu$ F. After 24 h of culture, the transfected cells were washed with PBS and used for luciferase assays, which were performed according to the vendor's instructions with the Dual-Luciferase<sup>®</sup> Reporter Assay System (Promega) in which the activities of

firefly (*Photinus pyralis*) and *Renilla* (*Renilla reniformis*) luciferases are measured sequentially from a single sample. Briefly, the cells were lysed in 100 µl 1x Passive Lysis Buffer for 15 minutes while shaking and 20µl of lysate were transferred to a F96 MicroWell™ Plate. After addition of 100 µl Luciferase Assay Reagent II (LAR II), firefly luciferase activity was measured, followed by addition of 100 µl Stop & Glo® Reagent and measurement of *Renilla* luciferase activity using a Centro 960LB luminometer. For data evaluation, firefly luciferase activity was normalized to the internal control luciferase activity (*Renilla*) to eliminate experimental variances such as differences in cell viability, transfection efficiency and cell lysis efficiency. All measurements were performed in duplicates.

### 2.2.2 Mice

General mouse work such as daily animal care, breeding and offspring separation was carried out in collaboration with the animal core facility of the Max-Delbrück-Center for Molecular Medicine, Berlin, Germany. All mice were housed and bred in specific pathogen-free animal facilities. All animal experiments were approved by the local authorities according to the German Federal Animal Protection Act.

#### 2.2.2.1 Mouse strains

Wildtype congenic B6.SJL-Ptprca (CD45.1<sup>+</sup>) mice were purchased from Taconic and were crossed with 129ola to obtain 129ola/B6.SJL (CD45.1<sup>+</sup>/CD45.2<sup>+</sup>) F1 generation animals used as recipients for BM cell transplantation. 129ola mice were used as wildtype counterparts for experiments.

*Dnmt1* knockdown mice (*Dnmt1*<sup>-chip</sup>), conditional *Dnmt1* knockout mice (*Dnmt1*<sup>lox/lox</sup>) and transgenic Mx1Cre mice have been described [Gaudet, et al., 2003; Jackson-Grusby, et al., 2001; Kuhn, et al., 1995]. *Dnmt1*<sup>-chip</sup> mice combine one *Dnmt1* null allele (deletion of exons 3 and 4) with a hypomorphic *Dnmt1* allele. This so called “chip” allele (cDNA homologous insertion protocol) was generated by inserting *Dnmt1* cDNA into the locus of the *Dnmt1* knockout allele [Tucker, et al., 1996]. A cDNA insertion in a knockout locus restores part of the original gene expression but since intron and exon structures are lost part of the regulatory network is lost and gene expression is reduced. In *Dnmt1*<sup>lox/lox</sup> mice, sequences encoding the catalytic domain of *Dnmt1* (exons 4 and 5) of *Dnmt1* are flanked by two loxP sites which are used for

conditional deletion of *Dnmt1*. In these mice, recombination (excision and consequently inactivation of the target gene *Dnmt1*) occurs only in those cells expressing the cyclization recombination protein (Cre recombinase). Cre-mediated excision of this flanked region causes an out-of frame splice yielding an mRNA encoding the first 67 of 1619 amino acids, thus lacking both the motifs for localization and the entire catalytic domain. Transgenic Mx1Cre mice express Cre recombinase under control of the IFN-inducible *Mx1* promoter which can be activated by application of Poly(I:C) [Kuhn, et al., 1995]. These strains were crossed to obtain *Mx1Cre<sup>+</sup>Dnmt1<sup>lox/chip</sup>* mice which representing inducible *Dnmt1* knockdown mice.

### 2.2.2.2 Genotyping

Mice were genotyped by locus-specific polymerase chain reaction (PCR) on genomic DNA extracted from tail tissue (see 2.2.1.5 and 2.2.1.1).

### 2.2.2.3 Dissection of mice and preparation of mouse organs

Mice were euthanized with CO<sub>2</sub>, rinsed with ethanol and pinned down on a dissecting board with the belly facing up. Mice were opened and the upper and lower hind leg as well as the front upper leg were dissected with scissors and forceps to isolate BM cells. Other organs (spleen, lymph nodes, sternum or thymus) were dissected when needed. All organs were kept in cold PBS until preparation. Single cell suspensions were generated by cutting the organ into small pieces and subsequently filtering it through a cell strainer. BM was isolated by thoroughly flushing the bones with PBS. Peripheral blood of living mice was taken from the tail vein with canula treated with EDTA to avoid clotting of blood cells. Red blood cells were lysed by incubation the cells with 1 ml of ice-cold ACK buffer. Subsequently, cell were washed in PBS.

Bone marrow stroma cells were isolated by crushing the bones (front and hind legs) and subsequent depletion of bone marrow cells by vigorously flushing the bones. Bone chips were incubated in serum-free MEM Alpha Modified medium supplemented with 0.2% collagenase for 1 hour at 37°C on a rotator. Afterwards, stroma cells were separated from residual bone fragments by filtering through a 100µm cell strainer.

#### 2.2.2.4 Transplantation experiments

For all transplantation assays (except reverse transplantation experiments, see below) adult 129ola/B6.SJL (CD45.1<sup>+</sup>/CD45.2<sup>+</sup>) F1 generation animals were used as recipients (8-12 weeks of age). Prior to transplantation they were irradiated with a sub-lethal dose of 6 Gy total body irradiation with the 18-MeV photon beam of a linear electron accelerator with a dose rate of 0.18 Gy/min. Donor cells (CD45.2<sup>+</sup>) in desired numbers were resuspended in 200 µl sterile PBS and injected intravenously into the tail vein of the fixed animal.

In case of *Myc/Bcl2* transduced LSK cells,  $2 \times 10^4$  donor cells were injected into recipient animals. For *Notch1-IC* and *MLL-AF9* leukemia experiments,  $5 \times 10^4$  to  $1 \times 10^5$  infected cells (YFP<sup>+</sup>/GFP<sup>+</sup>) were transplanted into recipients. Mice which had received *MLL-AF9*; *MxCre<sup>+</sup>Dnmt1<sup>lox/chip</sup>* cells were subjected to Poly(I:C) treatment as described in 2.2.2.5. Recipient mice were sacrificed when visibly ill and leukemia infiltrated organs were investigated by FACS, used for further *in vitro* studies or transplanted into secondary recipients.

For secondary transplantations and limiting dilution assays the desired number of leukemia cells was re-transplanted into secondary recipients. Leukemia stem cell (LSC) frequencies were determined using the L-Calc software. Such calculations are based on the assumption that LSCs follow a Poisson distribution. The Poisson distribution is a discrete probability distribution, which describes the outcome of independent experiments according to a yes-or-no answer (two possible outcomes). Transferred to the limiting dilution experiment the two possible outcomes are leukemia development or no leukemia development.

For short-term leukemia-cell engraftment assays,  $10^7$  leukemia cells were transplanted into sub-lethally irradiated recipient mice. 24 h after transplantation, recipients were sacrificed and donor cell contribution in different hematopoietic organs. was examined.

For reverse leukemia-cell transplantations 5000 *Dnmt1<sup>+/+</sup> MLL-AF9* cells were mixed with  $1 \times 10^6$  *Dnmt1<sup>+/+</sup>* bone marrow cells, which provided support for the recipient animals. This cell mixture was transplanted into lethally irradiated (10.5 Gy) *Dnmt1<sup>-</sup>/chip*) or *Dnmt1<sup>+/+</sup>* mice.



#### 2.2.2.5 Poly(I:C) treatment

Mx1Cre mice harbor the Cre recombinase gene under the control of the *Mx1* promoter [Kuhn, et al., 1995]. This promoter is normally silent in mice but can be induced to high levels of transcription by administration of interferon alpha, interferon beta or synthetic double-stranded RNA (such as Poly(I:C)). When combined with a mutant, carrying a gene that is flanked by loxP recognition sites, the expression of Cre recombinase causes the excision and removal of flanked genetic region. Excision of *Dnmt1*<sup>lox</sup> alleles in transplantation-recipients of MLL-AF9 transduced *Mx1Cre*<sup>+</sup>*Dnmt1*<sup>lox/chip</sup> was induced by intraperitoneal injection of 300 µg Poly(I:C) in PBS/ mouse every other day for a total of five injections. Mice transplanted with MLL-AF9 infected *Mx1Cre*<sup>-</sup>*Dnmt1*<sup>lox/chip</sup> BM cells were used as controls for nonspecific Poly(I:C) effects. Excision efficiency was analyzed by PCR. The excised Δ-allele generated a 0.25 kilobase (kb) band and the non-excised loxP-flanked allele generated a 0.15 kb band.

#### 2.2.2.6 Histology: May-Grünwald-Giemsa Stain

2 x 10<sup>4</sup> to 1 x 10<sup>5</sup> bone marrow cells isolated from leukemic mice were resuspended in 100 µl PBS were attached to a object slide by centrifugation (800 rpm, 5 min,) using a cytocentrifuge. Object slides were air-dried and cells were fixed in methanol for 5 minutes. For staining procedure, cytopsin preparations were stained in May-Grünwald solution for 5 minutes, rinsed with water and incubate in Giemsa staining solution for 45 minutes. Object slides were rinsed in water again, air-dried and stained cells were analyzed with a Leica microscope.

### 2.2.3 Cell culture

#### 2.2.3.1 Thawing, general cultivation and freezing of cells

To be thawed cell lines and primary cells were thawed in a 37°C water bath and quickly washed in 10 ml PBS or the respective culture medium to remove DMSO. Cells were collected by centrifugation at 1200 rpm for 5 minutes at room temperature (RT) followed by removal of the supernatant by Pasteur pipette connected to a pump. The cell pellet was resuspended in fresh culture medium and cells were plated in an appropriate cell number on culture dishes.



For cultivation, cells were cultured in an incubator at 37 °C and 5 % CO<sub>2</sub> using the respective culture media. Passaging of adherent cells was performed as follows: The culture medium was completely removed from the plate, cells were washed with 1 x PBS , followed by an approximately 5-minute incubation with 0.05 % trypsin-EDTA at 37 °C to detach the cells. The detached cells were resuspended in an appropriate volume of media and collected by centrifugation (1200 rpm, 5 minutes, RT). The desired number of cells was transferred to new culture dishes. Suspension cells were collected by centrifugation and re-seeded in the desired density in new cell culture flasks or dishes.

For freezing of cells, they were harvested, resuspended in 1 or 2 ml of ice-cold cryo-medium (FCS with 10 % DMSO) and transferred into cryo-tubes. Subsequently, cryo-tubes were chilled to -80°C in styrofoam boxes and, in case of long-term storage, transferred to liquid nitrogen.

### 2.2.3.2 Assessment of cell number and cell viability

Harvested cells were collected by centrifugation (1200 rpm, 5 minutes, RT) and the cell pellet was resuspended in 1x PBS. To enumerate the cells, 10 µl of the cell suspension were mixed with trypan blue solution in dilutions ranging from 1:1 to 1:20. and shortly incubated. A small aliquot was transferred to the hemocytometer (Neubauer chamber) and checked for equal distribution of the cells in all four big quadrants. The cells in at least one large squares were counted and the concentration of cells was calculated as follows: cells per ml = cells/square multiplied by 10,000. Cells which had taken up the trypan blue solution (blue cells) were identified as non-viable cells and excluded during counting.

### 2.2.3.3 Cell lines

Plat-E cells are a retrovirus packaging cell line which was generated based on the 293T cell line [Morita, et al., 2000]. Plat-E cells were cultured in DMEM, supplemented with 10 % FCS and 1 x Penicillin/streptomycin (P/S) and were used for the production of retroviral supernatants (see 2.2.3.4). E86 cells [Markowitz, et al., 1988] are a NIH 3T3 derived murine cell line which produces high levels of the retroviral proteins *gag-pol* and *env*. They were kept in DMEM, supplemented with 10

% FCS and were used for the stable production of high-titer supernatants of the MSCV-MLL-AF9-IRES-GFP retrovirus (see 2.2.3.4). K562 cells, a human chronic myelogenous cell line [Lozzio and Lozzio, 1975], was kept in RPMI supplemented with 10% FCS and 1 x P/S and used for luciferase assays conducted with *Gata1*-promoter constructs. 416B cells are a murine myeloid progenitor cell line [Dexter, et al., 1979] and were kept in DMEM with 20 % FCS and 1 x P/S. PU1null cells are an immortalized cell line established from *Pu.1*<sup>-/-</sup> fetal liver cells [Walsh, et al., 2002] and were cultivated in DMEM supplemented with 20 % FCS, 1 x P/S and 10 ng/ml IL-3. Both 416B cells and PU1null cells were used to test the effects of pharmacological demethylation on the expression of myeloerythroid differentiation genes.

### 2.2.3.4 Production of viral supernatants and transduction of cells

Retroviral supernatants were generated by co-transfecting Plat-E cells with 10 µg of the respective retroviral construct, 10 µg of a gag-pol construct and 2 µg of an ecotropic env construct. Transfection was conducted using the Calcium Phosphate Transfection Kit according to instructions. Successful transfections were controlled by FACS when GFP-expression constructs were used. Viral supernatants were collected at 48, 72 and 96 h after transfection, filtered through 0.45 µm filters and stored at -20°C.

For infections with the MSCV-MLL-AF9-IRES-GFP retrovirus, a stable virus producing E86 cell line was generated as follows: Viral supernatant produced as described in the preceding passage were used to infect E86 cell. The infection was performed with a 1:1 mixture of DMEM medium and viral supernatant, supplemented with 8µg/ml polybrene for 48 hours. After infection, the cells were collected and infected cells, identifiable by the expression of GFP, were FACS sorted. GFP+ cells (further on designated as E86-MLL-AF9-IRES-GFP) were expanded and frozen until used for the infection of hematopoietic cells.

For retroviral transduction of LSK cells or c-Kit enriched cells with the MSCV-c-Myc-IRES-Bcl2 retroviral construct, cells were pre-stimulated for 24 h at 37°C in medium (IMDM, 20 % FCS, 100 µg/ml of penicillin, 2 mM L-glutamine) containing 50 ng/ml SCF, 20 ng/ml IL-6 and 20 ng/ml Lif. Subsequently, cells were mixed with retroviral supernatants in the presence of 50 ng/ml SCF, 20 ng/ml IL-6, 20 ng/ml LIF and 8µg/ml polybrene and cultured for 48 h at 37°C.

Infections with the MSCV-MLL-AF9-IRES-GFP retrovirus were performed as a 4 co-culture of E86-MLL-AF9-IRES-GFP and the to be infected bone marrow cells which had been pre-stimulated for 24 hours as described in the previous passage. The co-culture was conducted for 48 h in IMDM, 20 % FCS, 100 µg/ml of penicillin, 2 mM L-glutamine supplemented with 50 ng/ml SCF, 20 ng/ml IL-6, 20 ng/ml LIF and 8µg/ml polybrene. After infection, a small aliquot of cell served to determine the infection rate by FACS.

Infected cells were either transplanted in mice (2.2.2.4) or used for *in vitro* colony assays in methylcellulose (2.2.3.5) .

Retroviral infections of PU1null cells with MSCV-Gata1-IRES-GFP or MSCV-IRES-GFP control construct were carried out for 48 h in viral supernatant, supplemented with 10 ng/ml IL-3 and 8µg/ml polybrene. Infection rates were determined by FACS.

### 2.2.3.5 Serial replating assay in Methylcellulose

Serial replating assays with MLL-AF9 transformed bone marrow cells was performed similarly to previously described [Somervaille and Cleary, 2006]. C-kit enriched bone marrow cells were infected with the MSCV-MLL-AF9-IRES-GFP retrovirus or MSC-IRES-GFP virus as control. Infected cells were FACS sorted and  $10^4$  cells were plated in methylcellulose medium (M3234, Stem Cell Technologies) supplemented with 20 ng/ml SCF, 10 ng/ml IL-3, 10 ng/ml IL-6, and 10 ng/ml GM-CSF. 1 ml of this suspension was plated onto a 35 mm cell culture dish and three of these dishes were put into one 150 mm cell culture dish together with one dish filled with water to prevent drying of the semi-solid media. After 5 days, colonies were counted and cells harvested from the plates. 5,000 MLL-AF9 transformed cells were re-seeded as described before and colony numbers were again scored after 5 days. 4 rounds of replating were performed with triplicates for each sample.

### 2.2.3.6 5-aza-2'-deoxycytidine treatment

416B cells, PU1null cells and primary leukemic *MLL-AF9* blasts were treated with 5-aza-2'-deoxycytidine (5-aza) for 72 h. Whereas 416B cells and MLL-AF9 blasts received a concentration of 5 µM 5-aza, 1 µM was used for PU1 null cells. Medium of control cells was supplemented with the equal volumes of solvent (50 % acetic acid).

Medium was exchanged after 48 h and cells were supplied with fresh medium and 5-aza/solvent. After 72 h cells were harvested and RNA was extracted as described in 2.2.1.2 or subjected to FACS analysis (only 416B).

## **2.2.4 Fluorescence activated cell sorting (FACS)**

### *2.2.4.1 General flow cytometry and cell sorting.*

Single cell suspensions were immuno-stained with various fluorescence-conjugated antibodies listed in chapter 2.1.9. Staining was performed in PBS with the fluorochrome-conjugated antibodies usually using a 1:100 dilution for each antibody. Samples were incubated in the fridge for 20-40 minutes and subsequently washed with PBS to remove excess antibody. In case biotinylated antibodies were used, streptavidin fused to a fluorochrome was added in a 1:100 dilution for a secondary staining step, followed by a 15-minute incubation in the fridge and subsequent washing. Cells were resuspended in FACS buffer and fluorescence intensity was measured with a FACSCalibur cytometer equipped with CellQuestPro software or a LSRII/Fortessa cytometer and Diva software. Discrimination of dead cells was accomplished by staining with propidium iodide (PI) or (DAPI) just before measurement. Data analysis was performed with FlowJo software

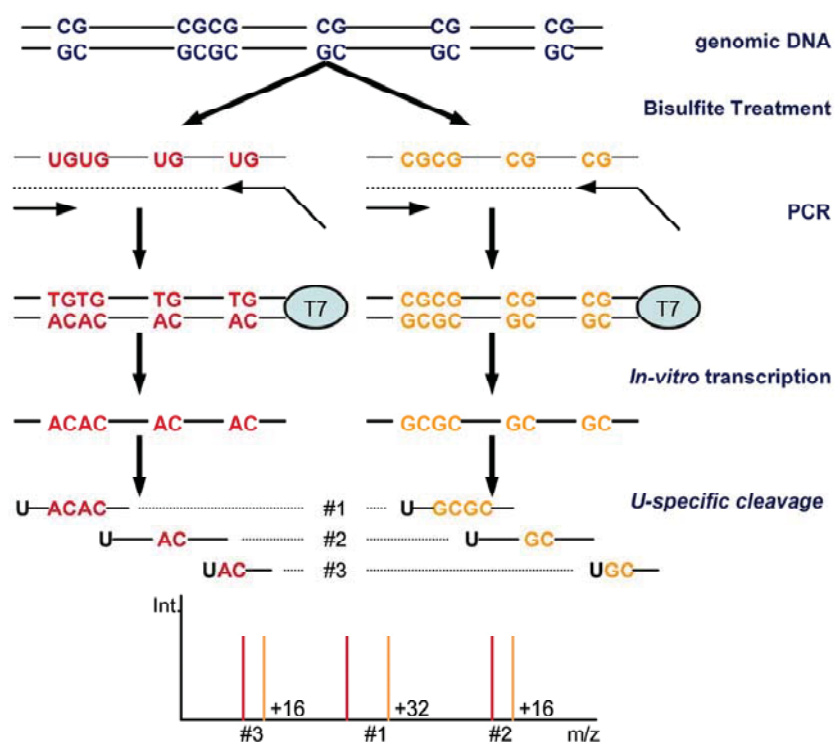
For sorting of hematopoietic stem cells (HSCs), here defined as  $\text{lin}^{-/\text{low}}\text{Sca-1}^+\text{c-Kit}^+$  [Ikuta and Weissman, 1992; Spangrude, et al., 1988], bone marrow cells were pre-selected by lineage depletion which was achieved with a lineage “cocktail” of antibodies against CD3 $\epsilon$ , CD4, CD8 $\alpha$ , B220, CD19 and Gr-1 (all monoclonal rat IgGs). Subsequently, cells positive for these markers were depleted with immuno-magnetic beads conjugated to polyclonal sheep-anti-rat IgG antibodies. Lineage depleted cells ( $\text{Lin}^-$ ) were stained with the respective antibodies and cell sorting was done with a high-speed multicolor cell sorter (FACSAria) using standard protocols. For enrichment of stem- and progenitor cells used for infection, either lineage-negative cells ( $\text{Lin}^-$ ) were used or c-Kit-enrichment was performed with CD117 microbeads. Before sorting, cells were filtered with a cell strainer.

#### 2.2.4.2 Cell cycle analysis

Propidium iodide (PI) binds to DNA by intercalating between the bases without sequence preferences which makes it a suitable dye to quantify the DNA content of a cell. This characteristic allows to use PI for the determination of the cell cycle stage (G1-S/G2/M-Phase) and also displays a rough method to examine the percentage of dead apoptotic cell, which have a DNA content below 2n (Sub-G1). For PI staining cells were either directly FACS sorted into 70 % ethanol or cultured cells were harvested and added drop wise to pre-cooled ethanol while vortexing. After fixation of cells, they were incubated for 15 minutes on ice, followed by the addition of the PI solution at a final concentration of 50 µg/ml. Stained cell were measured by means of flow cytometry at a maximum emission wavelength of 617 nm.

#### 2.2.5 MassARRAY

The quantitative analysis of DNA methylation at single CpG units was performed by MassARRAY as previously described [Ehrich, et al., 2005] and schematically described in **Figure 9**. Therefore, 500 ng (sorted primary cells) or 1 µg (cell lines) of genomic DNA was treated with sodium bisulfite, PCR-amplified, *in vitro* transcribed, cleaved by RNase A and subjected to matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Methylation standards (0, 20, 40, 60, 80 and 100 % methylated genomic DNA) and correction algorithms based on the R statistical computing environment were used for data normalization. The genomic localization of the analyzed regions in the *Gata1* and *Cd48* locus are the following: *Gata1*, chr. X 7545431–7545769 and chr. X 7553755–7554238; *Cd48*, chr. 1 173612289–173612577.



**Figure 9: Concept of the MassARRAY technology**

Genomic DNA undergoes bisulphite treatment and PCR amplified using primers located outside of the CpG island with one primer tagged with a T7 promoter sequence. The PCR product is *in vitro* transcribed to RNA, which is then cleaved in a base-specific manner. Analysis of the cleavage products is performed with MALDI-TOF mass spectrometry.

### 2.2.6 Statistical analysis

In order to determine the statistical significance of experimental results Student's t-tests were conducted. \* $P \leq 0.05$  and \*\* $P \leq 0.001$

### 3 Results

To address the question how leukemia stem cell function and fate choices are affected by DNA methylation, a genetic knockdown mouse model for the main somatic methyltransferase DNMT1 was used. This mouse model was chosen, as prior observations suggested the assumption that a full knockout of *Dnmt1* would cause rapid cell death of leukemic cells, consequently not allowing a thorough functional analysis of LSCs in the cause of leukemia. First, studies in our lab revealed that deletion of *Dnmt1* caused a complete failure of the hematopoietic system due to rapid cell death of all hematopoietic cells [Broske, et al., 2009]. Secondly, complete inactivation of *Dnmt1* in human cancer cells led to mitotic catastrophe in these cells followed by cell death [Chen, et al., 2007]. Thirdly, preliminary *in vitro* studies were conducted in our lab utilizing retrovirally introduced Cre-recombinase into *Dnmt1<sup>lox/lox</sup>* conditional *Dnmt1* knockout leukemia cells (C. Langhans, L. Vockentanz and F. Rosenbauer, unpublished results). These experiments have shown rapid cell death of myeloid leukemia cells upon *Dnmt1* deletion (data not shown). Based on this knowledge we used the hypomorphic *Dnmt1* mouse model created in Rudolf Jaenisch's group [Gaudet, et al., 2003]. These *Dnmt1* knockdown mice are termed *Dnmt1<sup>-chip</sup>* and have been introduced in chapters 1.4.3 and 2.2.2.1. Reduced *Dnmt1* expression has been shown in various *Dnmt1<sup>-chip</sup>* cell types, which also included hematopoietic cells. Furthermore, genomic hypomethylation has been demonstrated in hematopoietic and other tissues [Broske, et al., 2009; Gaudet, et al., 2003]. *Dnmt1<sup>-chip</sup>* mice and an inducible variant of the *Dnmt1* knockdown mouse model were applied to investigate the role of *Dnmt1* in leukemogenesis and the results of these studies are presented in the following chapters.

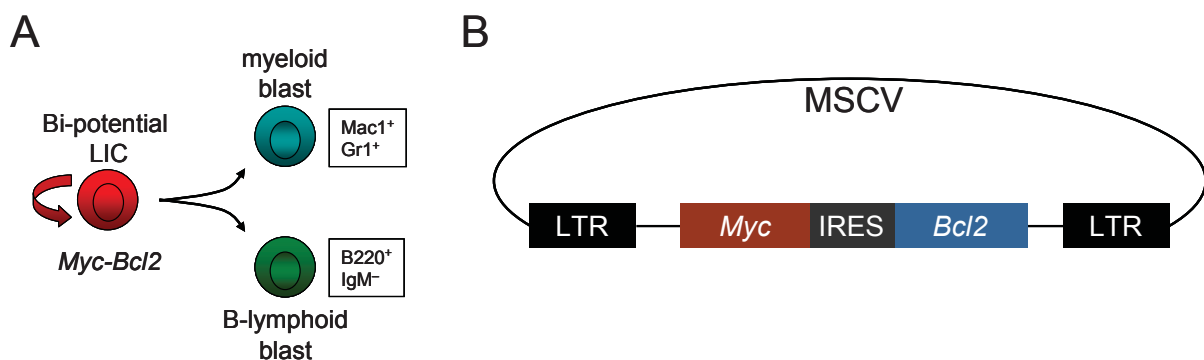
#### 3.1 DNA methylation controls lineage choices of leukemia initiating cells

##### 3.1.1 *Myc-Bcl2* induced leukemia in lineage negative cells

In order to investigate how hypomethylation affects the lineage differentiation choices of leukemia initiating cells, I applied a leukemia model, which encompasses both



major hematopoietic branches, the lymphoid and the myeloid lineage. A leukemia model following these characteristics was known to be achieved by ectopic expression of *c-Myc* and *Bcl2*. *c-Myc* is a classical oncogene which acts as a pro-proliferative transcription factor frequently involved in malignant transformation upon its overexpression [Dudley, et al., 2002]. *Bcl2* was first isolated in B-cell lymphomas, hence the name *B-cell lymphoma 2*, and it acts as an oncogene by repression of cell death [Chao and Korsmeyer, 1998; Vaux, et al., 1988]. Co-expression of these two oncogenes was shown to induce a bilinear myeloid/B-lymphoid leukemia in wildtype mice [Luo, et al., 2005] thus representing a model encompassing both acute myeloid leukemia (AML) and acute (B-)lymphoid leukemia (B-ALL). LSCs in such a bilinear disease can be described as bi-potential and have to carry out a lineage decision either contributing to the myeloid or the B-lymphoid blast population (**Figure 10A**). *Myc-Bcl2* co-expression was achieved by infection of hematopoietic cells with a murine retrovirus, the construct for which is depicted in **Figure 10B**.



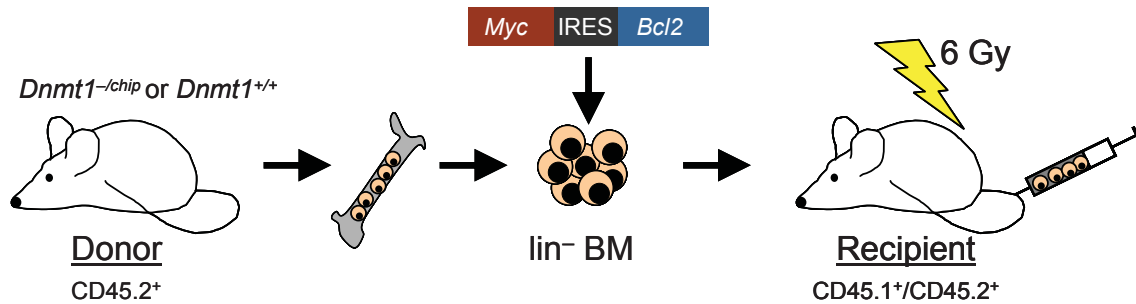
**Figure 10: *Myc* and *Bcl2* co-expression construct induces bilinear leukemia**

**A)** The co-expression of *Myc* and *Bcl2* creates a bi-potential leukemia initiating cell (LIC) which differentiates into either a myeloid or a lymphoid blast. Surface markers describing the two blast populations are depicted in the black boxes. **B)** A murine retroviral MSCV (Murine Stem Cell Virus) vector was used for leukemic transformation. Bicistronic *Myc-Bcl2* mRNA expression is driven by LTR (long terminal repeat) sequences. IRES, internal ribosomal entry site.

In order to generate a *Myc-Bcl2* driven leukemia in an either wildtype or hypomethylated background, progenitor enriched bone marrow cells ( $\text{lin}^-$  BM) from either *Dnmt1*<sup>-chip</sup> or *Dnmt1*<sup>+/+</sup> mice were infected with a MSCV-*Myc-Bcl2* retrovirus. After infection, cells were transplanted into sublethally irradiated recipient mice by intravenous injection (**Figure 11**). As hematopoietic cells from donors and recipients



carry different variants of the leukocyte common antigen CD45 they can be distinguished by FACS analysis, allowing to monitor leukemia development in the recipient mice by analysis of peripheral blood samples.



**Figure 11: Experimental setup for *Myc-Bcl2* driven leukemia**

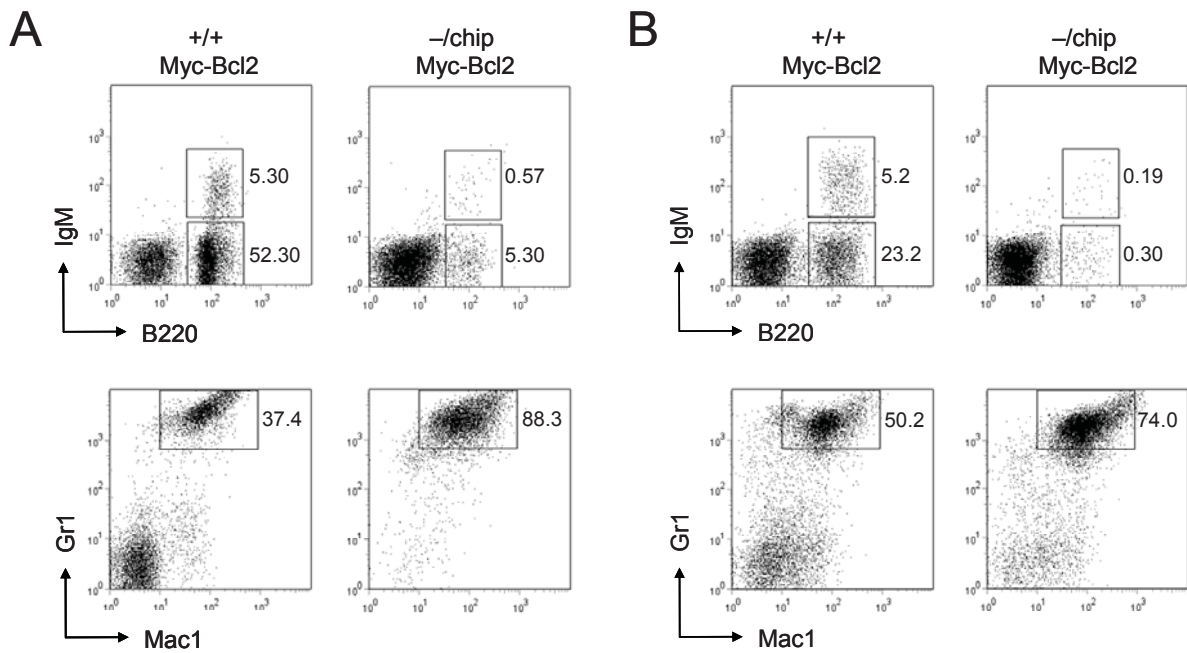
Bone marrow cells from CD45.2<sup>+</sup> donor mice were isolated and lineage-positive cells were depleted. Lineage negative cells (*lin*<sup>-</sup>) were infected with *Myc-Bcl2* retrovirus and infected cells were transplanted into CD45.1<sup>+</sup>/CD45.2<sup>+</sup> SJLx129ola recipient mice, which had previously received a sublethal irradiation dose of 6 gray (Gy). BM, bone marrow.

Both, recipient mice which had received transformed *Dnmt1*<sup>-/-chip</sup> cells and those which had received *Dnmt1*<sup>+/+</sup> cells developed leukemia, characterized by strongly elevated white blood cells counts and enlarged spleen and lymph nodes as summarized in **Table 1**. Latency times until mice succumbed to leukemia was  $24.0 \pm 2.4$  days post-transplantation for recipients of *Dnmt1*<sup>+/+</sup> *Myc-Bcl2* cells and  $30.7 \pm 7.0$  days for recipients of *Dnmt1*<sup>-/-chip</sup> *Myc-Bcl2* cells. Leukemic cells from infiltrated organs were analyzed by FACS (**Figure 12**). This analysis revealed that *Dnmt1*<sup>+/+</sup> *Myc-Bcl2* leukemias, as expected, consisted of two major blast populations. On the one hand, myeloid blast cells, characterized as Mac1<sup>+</sup>/Gr1<sup>+</sup>, were present. On the other hand an about equally strong lymphoid cell population was found. The lymphoid cells were characterized by the B-cell marker B220 and the lack of Immunoglobulin M (IgM) on these cells confirmed their immature nature. Contrasting to that, this B-lymphoid leukemia cell population was virtually absent in *Dnmt1*<sup>-/-chip</sup> *Myc-Bcl2* leukemias, which almost exclusively consisted of myeloid blasts. These findings suggest that hypomethylated LSCs have greatly diminished ability to form a B-ALL, however they are still capable of generating an AML.

**Table 1: Phenotype of *Dnmt1*<sup>+/+</sup> and *Dnmt1*<sup>-/-chip</sup> *Myc-Bcl2* leukemias.**

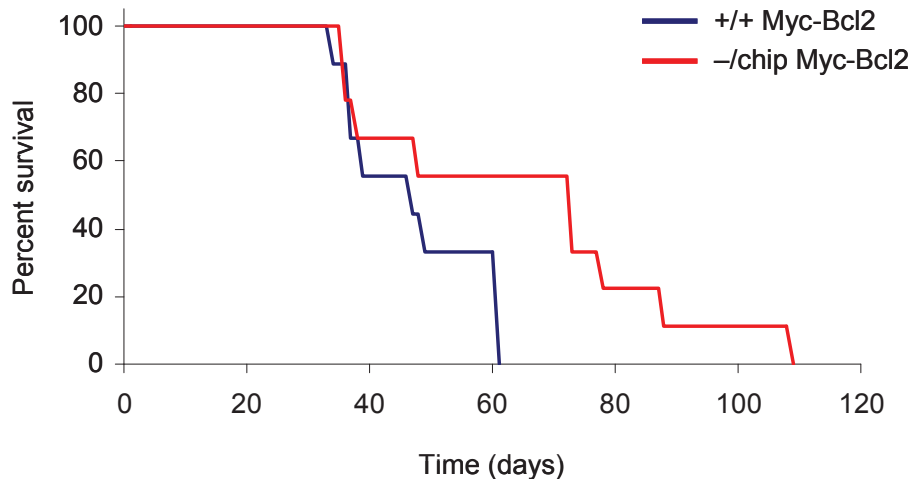
This table summarizes data from *Myc-Bcl2* leukemic animals from experiments shown in chapters 3.1.1 and 3.1.2 (n=16-17). Parameters collected from non-leukemic wildtype mice are shown for comparison. WBC, white blood cell count.

	<i>Dnmt1</i> <sup>+/+</sup> <i>Myc-Bcl2</i>	<i>Dnmt1</i> <sup>-/-chip</sup> <i>Myc-Bcl2</i>	Non-leukemic control ( <i>Dnmt1</i> <sup>+/+</sup> )
WBC (10 <sup>3</sup> /μl)	178.1 ± 137.7	126.5 ± 151.5	12.8 ± 5.6
Spleen weight (g)	0.68 ± 0.22	0.80 ± 0.34	0.12 ± 0.02
Lymph node weight (mg)	25.9 ± 7.7	27.1 ± 13.3	3.9 ± 1.9

**Figure 12: Characterization of *Myc-Bcl2* induced leukemias by FACS**

FACS analysis of leukemias given rise by transduced *Dnmt1*<sup>-/-chip</sup> and *Dnmt1*<sup>+/+</sup> cells. Mice were autopsied when visibly ill and organs were FACS analyzed in respect to the lineage composition. FACS analysis of transplantation donor cells (CD45.2<sup>+</sup>/CD45.1<sup>-</sup>) is shown for **A**) bone marrow and **B**) peripheral blood. The lymphoid blast population is depicted in the upper row of plots characterized as B220<sup>+</sup> and mostly IgM<sup>+</sup>. Myeloid blast cells (bottom row) are Gr1<sup>+</sup>Mac1<sup>+</sup>. One representative mouse from each genotype is shown. Numbers indicate percentages within the respective gates.

In order to confirm the transformed nature of *Dnmt1*<sup>-/chip</sup> and *Dnmt1*<sup>+/+</sup> *Myc-Bcl2* cells I transplanted leukemic cells from diseased mice into secondary recipients. As depicted in **Figure 13** all secondary recipients succumbed to leukemia, demonstrating the malignancy of the transplanted cells.



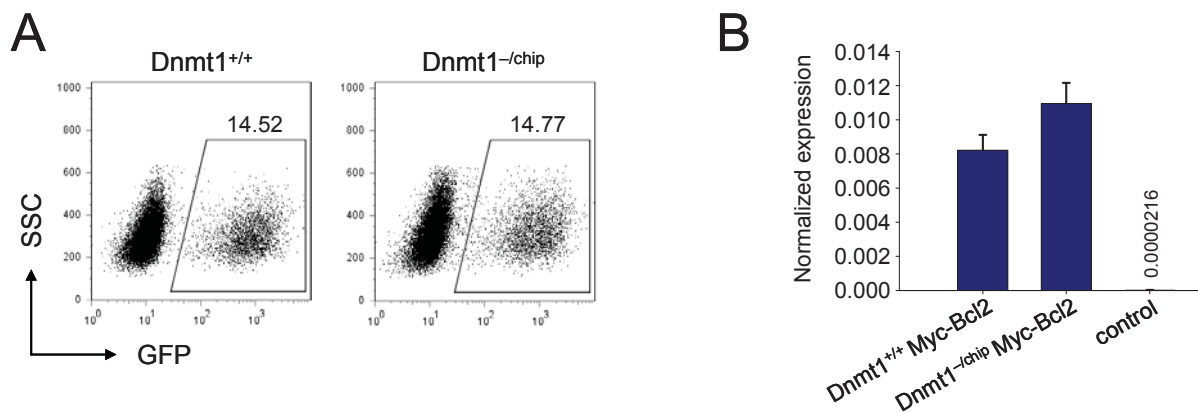
**Figure 13: Secondary transplantation of *Myc-Bcl2* blasts**

Death curve of secondary recipients re-transplanted with  $2 \times 10^5$  leukemia cells isolated from *Dnmt1*<sup>-/chip</sup> or *Dnmt1*<sup>+/+</sup> *Myc-Bcl2* leukemias. In total  $n = 9$  for each genotype (including each 3 recipients of leukemic splenocytes, thymocytes or lymph node cells.)

### 3.1.2 *Myc-Bcl2* leukemia from transformed stem cells

The results presented in the previous section suggested that an actually bi-potential leukemia initiating cell is not able to give rise to B-lymphoid blasts in a hypomethylated background. However, there is the possibility that the different phenotypes of *Dnmt1*<sup>+/+</sup> *Myc-Bcl2* and *Dnmt1*<sup>-/chip</sup> *Myc-Bcl2* leukemias were due to differences in the cell population which was used for the initial infection. Our previous studies have revealed that *Dnmt1*<sup>-/chip</sup> mice lack progenitor cells with generally lymphoid or specifically B-cell commitment, such as common lymphoid progenitors (CLPs), Pro- and Pre B-cells [Broske, et al., 2009]. Consequently, the *Dnmt1*<sup>-/chip</sup> cells used for transduction might already have a certain bias for choosing a myeloid fate, whereas *Dnmt1*<sup>+/+</sup> cells are susceptible for both lineages. In order to rule out such initial transduction biases I repeated this experiment, however, this time choosing purified stem cells for the *Myc-Bcl2* transduction, representing cells which

have not undergone a lineage decision so far. Before performing this experiment, I tested whether hypomethylated stem cells are equally infectable and whether they expressed the retroviral construct at similar rates. As the *MSCV-Myc-Bcl2* construct does not feature a fluorescent tag, such as GFP, allowing easy quantification of infection rates by FACS analysis, infectability was determined in a parallel infection with a non-oncogenic *MSCV-IRES-GFP* vector. As depicted in **Figure 14A** both *Dnmt1*<sup>-chip</sup> and *Dnmt1*<sup>+/+</sup> stem cells showed very similar infection rates, demonstrated by about equal numbers of *GFP*<sup>+</sup> cells. Expression level of the retrovirally introduced genes was assessed by real-time RT-PCR, which confirmed similar expression of the *Myc-Bcl2* bicistronic mRNA (**Figure 14B**).

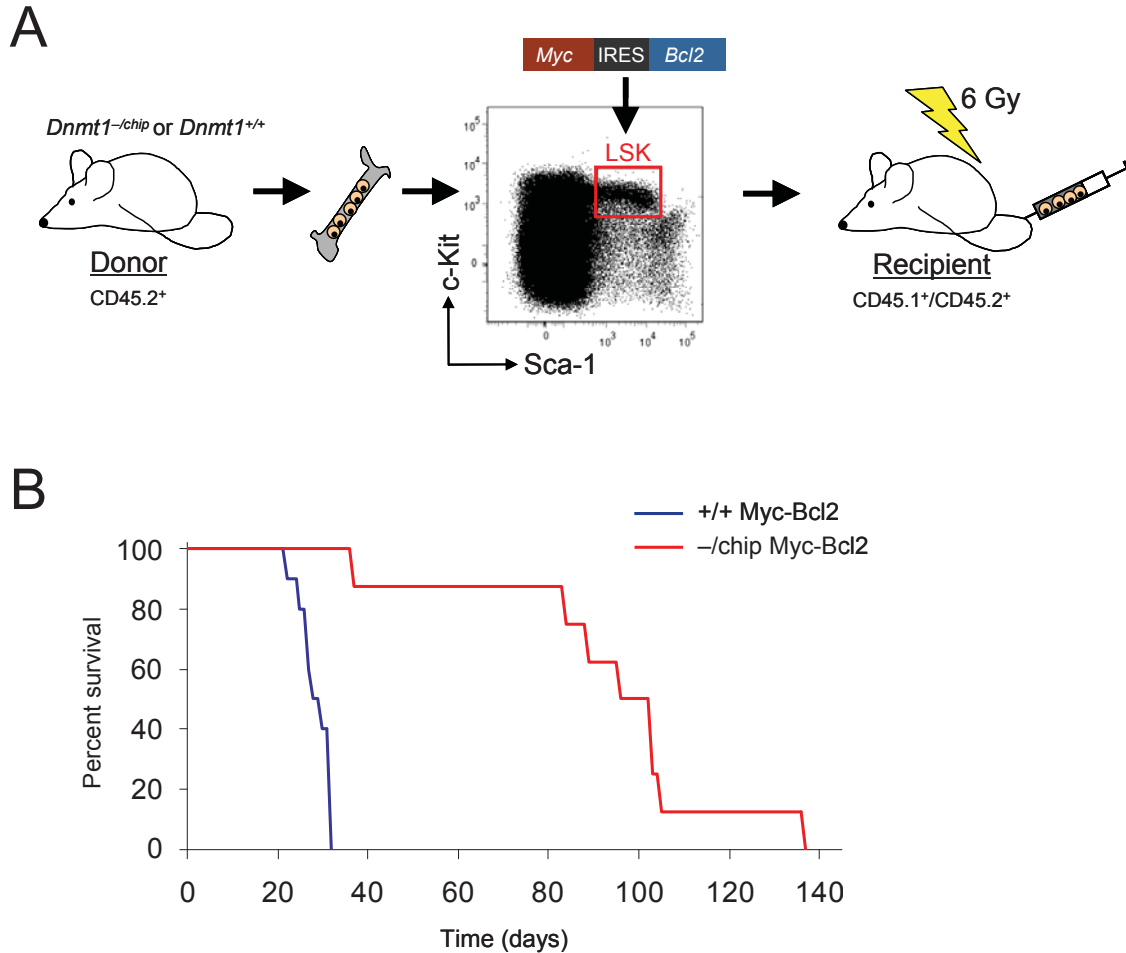


**Figure 14: Testing stem cell infectability and oncogene expression**

**A)** Sorted *Dnmt1*<sup>-chip</sup> and *Dnmt1*<sup>+/+</sup> stem cells (LSK) were infected with a *MSCV-IRES-GFP* retrovirus. 48 hours after infection, cells were harvested and the proportion of *GFP*<sup>+</sup> (=infected) cells was determined by FACS analysis. Percentages of gated cells are indicated. SSC, Sideward Scatter. **B)** *Dnmt1*<sup>-chip</sup> and *Dnmt1*<sup>+/+</sup> LSK cells infected with *MSCV-Myc-Bcl2* were harvested 48 hours after infection, RNA was isolated and transcription levels were determined by real time PCR after reverse transcription. Data were normalized to *Gapdh* expression. Non-infected cells served as negative control.

To transduce stem cells with the retroviral *Myc-Bcl2* construct, LSK cells were sorted from bone marrow of *Dnmt1*<sup>-chip</sup> or *Dnmt1*<sup>+/+</sup> mice according to the gating depicted in **Figure 15A** and processed as described in the experimental setup. Leukemia development was monitored by blood analysis and moribund mice were sacrificed and analyzed. As in the previous experiment, all recipient mice succumbed to leukemia, however, mice, which had received *Dnmt1*<sup>-chip</sup> *Myc-Bcl2* cells developed

leukemia with significantly longer latencies (**Figure 15B**). Median survival was  $27.7 \pm 3.5$  days for  $Dnmt1^{+/+}$  and  $93.3 \pm 28.1$  days for  $Dnmt1^{-/chip}$  ( $P < 0.0001$ ).

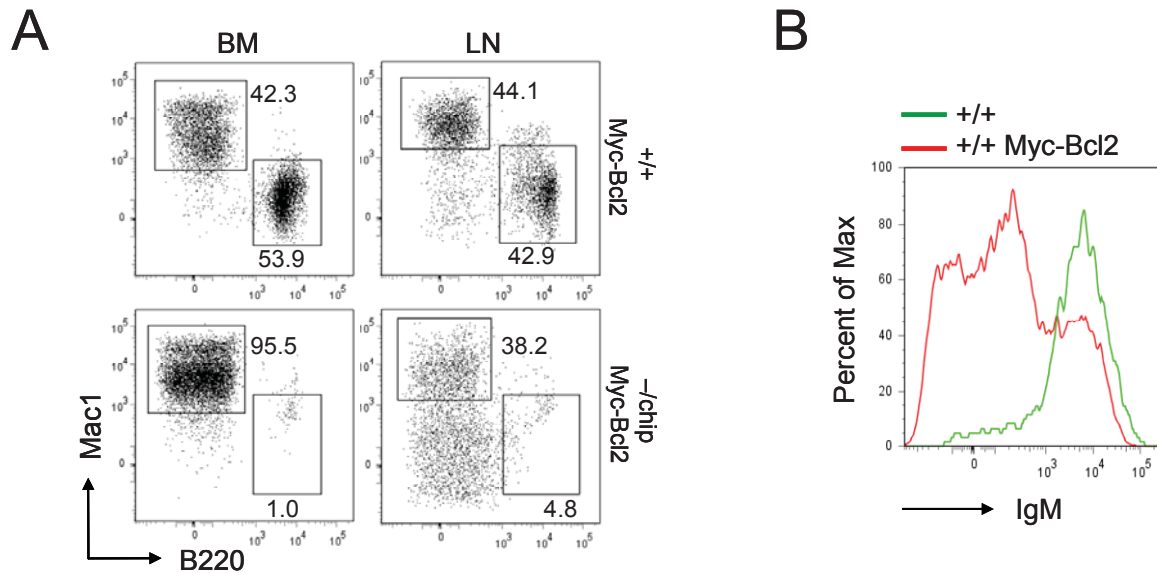


**Figure 15: FACS sorting of LSK cells and experimental setup**

**A)** LSK cells were FACS sorted from  $Dnmt1^{-/chip}$  or  $Dnmt1^{+/+}$  bone marrow cells as  $Sca-1^{+}c-Kit^{+}$  cells. The FACS plot shows cells within  $lin^{-}$  gate. After retroviral transduction with *Myc-Bcl2* cells were intravenously transplanted into sublethally irradiated recipient mice. **B)** Death curve of  $Dnmt1^{-/chip}$  and  $Dnmt1^{+/+}$  *Myc-Bcl2* leukemic animals induced by transplantation of  $10^4$  transduced cells.  $n=11$  ( $Dnmt1^{+/+}$ ) and  $n=8$  ( $Dnmt1^{-/chip}$ )

$Dnmt1^{+/+}$  *Myc-Bcl2* leukemias displayed an about equal distribution of myeloblasts and lymphoblasts (**Figure 16B**, upper row). The immature and leukemic nature of  $Dnmt1^{+/+}$  *Myc-Bcl2* B-cell blasts was again demonstrated by their lack of IgM expression in the spleen, compared to residual recipient B-cells in that organ (**Figure 16C**). As observed before,  $Dnmt1^{-/chip}$  *Myc-Bcl2* leukemias were almost completely

devoid of lymphoblast and almost exclusively consisted of Mac1<sup>+</sup> myeloid leukemia cells (**Figure 16B**, bottom row).

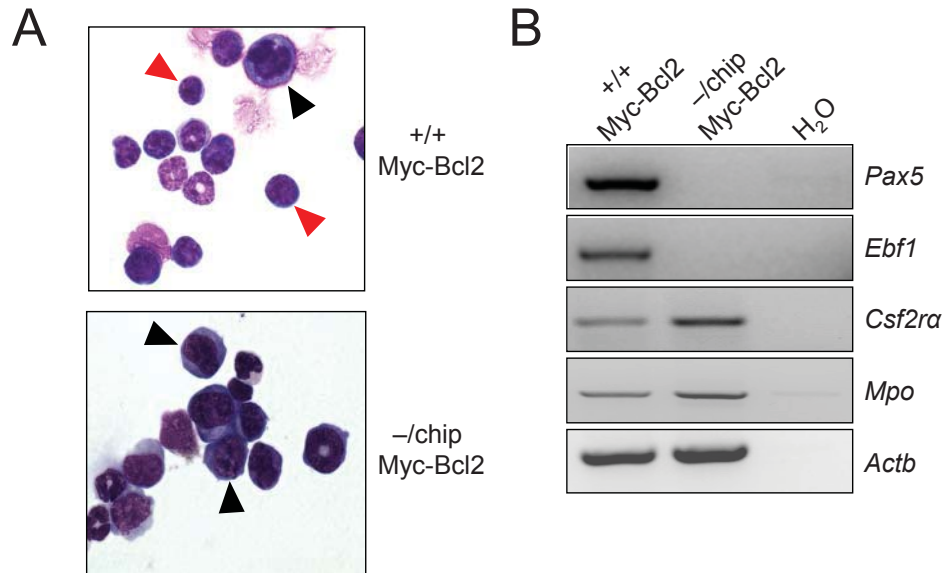


**Figure 16: Hypomethylation in LSCs blocks lymphoid blast development I**

**A)** Representative FACS plots of bone marrow (BM) and lymph node (LN) suspensions from diseased mice demonstrate loss of the neoplastic B cell population in leukemic mice reconstituted with *Dnmt1*<sup>-/-chip</sup> *Myc-Bcl2* cells. Plots show cells within donor (CD45.2<sup>+</sup>) gates. **B)** Immunoglobulin M (IgM) expression on *Dnmt1*<sup>+/-</sup> *Myc-Bcl2* B cell leukemic blasts (CD45.2<sup>+</sup>B220<sup>+</sup>) in recipient spleens. For comparison recipient splenic B cells (CD45.1<sup>+</sup>CD45.2<sup>+</sup>B220<sup>+</sup>) are shown.

As leukemia cells frequently display aberrant cell surface marker expression, it is necessary to confirm the absence of B-lymphoid blasts in hypomethylated leukemias by other methods than FACS. To this end, *Myc-Bcl2* tumor cells were investigated for their morphology. Cytospin preparations of leukemic bone marrow cells demonstrated the presence of both myelo- and lymphoblasts in wildtype leukemias, the latter ones, however, were absent in the *Dnmt1*<sup>-/-chip</sup> leukemias (**Figure 17A**). Furthermore, leukemia cells were analyzed for their expression of genes indicative of either myeloid or lymphoid cell identity. *Mpo*, the gene encoding for myeloperoxidase and *Csf2ra*, encoding for the granulocyte-macrophage colony-stimulating factor (GM-CSF) receptor were chosen as myeloid-specific genes, whereas *Pax5* (Paired box protein 5) and *Ebf1* (Early B cell factor 1) were selected as genes specific for the B-lymphoid lineage. RT-PCR revealed expression of *Mpo* and *Csf2ra* in both

leukemias. In contrast, *Pax5* and *Ebf1* transcripts were exclusively found in *Dnmt1*<sup>+/+</sup> leukemia cells (**Figure 17B**).



**Figure 17: Hypomethylation in LSCs blocks lymphoid blast development II**

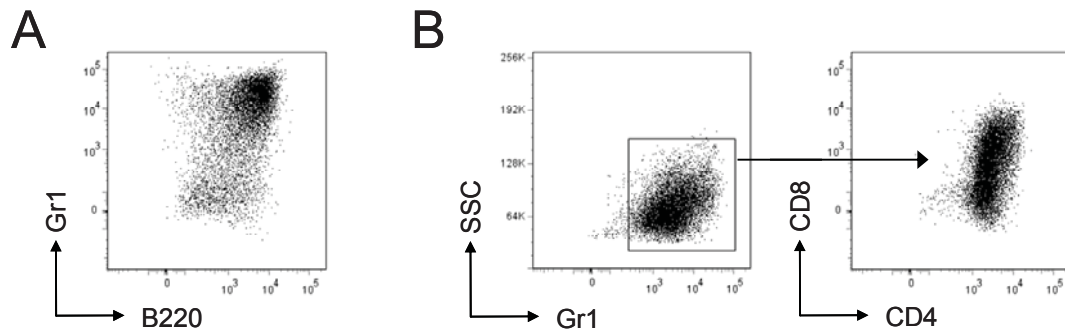
**A)** Cytopsin preparations of leukemic bone marrow cells confirming absence of B-cell blasts (red arrowheads) and presence of myeloblasts (black arrowheads) in *Dnmt1*<sup>-/-chip</sup> leukemic mice (May-Gruenwald stain, ×1,000 magnification). **B)** Genes indicative of myeloid cells (*Mpo*, myeloperoxidase; *Csf2ra*, granulocyte-macrophage colony-stimulating factor (GM-CSF) receptor-α) were expressed in leukemic bone marrow samples of moribund *Dnmt1*<sup>-/-chip</sup> *Myc-Bcl2* LSK cell-reconstituted mice, whereas expression of genes indicative of B cells (*Pax5*, *Ebf1*) was not detectable. Shown is one of two independent RT-PCR experiments with similar outcomes. Donor-derived blast cell infiltration in recipient bone marrow was >95% in all cases.

### 3.1.3 *Myc-Bcl2* leukemia with aberrant immunophenotype

As mentioned above, tumor cells frequently show abnormal patterns of cell surface marker expression due to non-physiological up- or down-regulation of the respective genes. This causes aberrant immunophenotypes which are usually not present on normal cells, as for example simultaneous expression of T-lymphoid and myeloid markers as well as B-lymphoid and myeloid markers. These kinds of abnormal combinations are for instance often found in cases of infant leukemias, which are consequently termed mixed lineage leukemia [Rubnitz, et al., 2009]. Aberrant co-expression of B-lymphoid and myeloid markers was observed in rare cases of both



primary and secondary *Dnmt1*<sup>-chip</sup> and *Dnmt1*<sup>+/+</sup> *Myc-Bcl2* leukemias (exemplarily depicted in **Figure 18A** for *Dnmt1*<sup>-chip</sup> *Myc-Bcl2*). Furthermore, I observed co-expression of T-cell and myeloid markers in 2 out of 8 *Dnmt1*<sup>-chip</sup> leukemias (**Figure 18B**).



**Figure 18: Aberrant immunophenotypes of *Myc-Bcl2* leukemias**

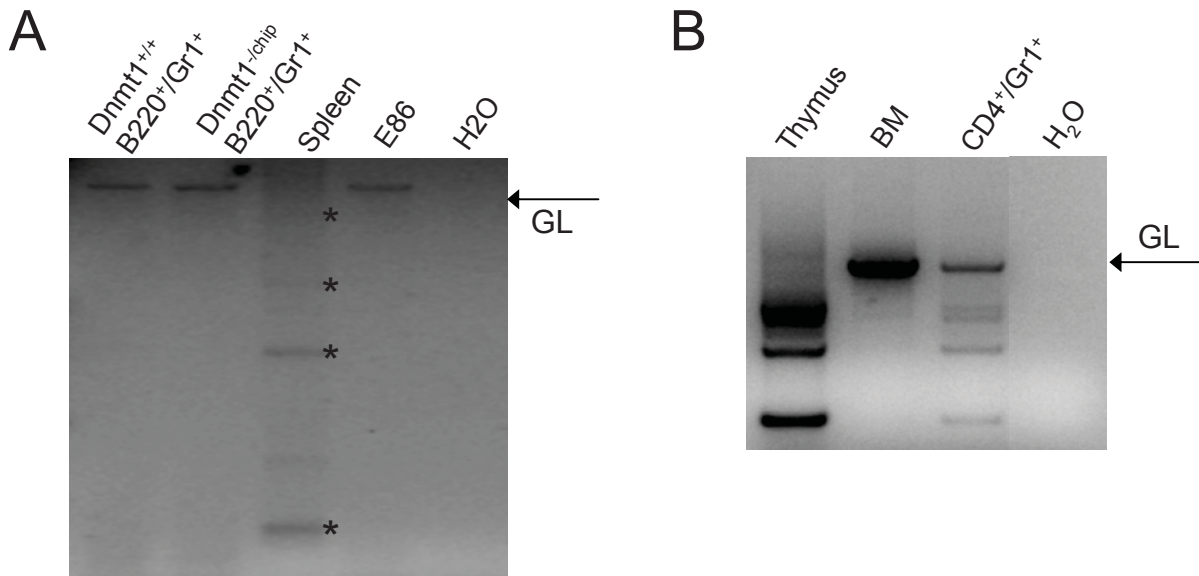
**A)** *Dnmt1*<sup>-chip</sup> bone marrow blasts of simultaneously expressing myeloid (Gr1) and B-lymphoid markers (B220). Cells shown are gated on donor cells. **B)** *Dnmt1*<sup>-chip</sup> bone marrow blast are positive for Gr1 (left plot) and also show expression of the T-cell markers CD4 and, in part, CD8 (right plot).

In order to test whether Gr1<sup>+</sup>/B220<sup>+</sup> leukemia cells rather represent a myeloid blast population with aberrant B220 expression or vice versa, the cells were tested for their genomic immunoglobulin rearrangement status. B-lineage cells reassemble gene segments in the immunoglobulin (Ig) locus to create a great variety of Ig molecules to mediate humoral immunity. These alterations are passed on through cell generations as they display a change in the genomic sequence. This unique feature allows to identify cells, which once belonged to the B-lymphoid lineage, but display characteristics (e.g. surface markers) not associated with B-cell identity later on. To this end I tested the rearrangement status of Gr1<sup>+</sup>/B220<sup>+</sup> leukemia cells by PCR (**Figure 19A**). In contrast to normal spleen cells which displayed a pattern of different rearrangement combinations, Gr1<sup>+</sup>/B220<sup>+</sup> blasts showed germ line configuration of the Ig locus indicating that these cells do not originate from the B-cell lineage, but merely aberrantly express a B-cell marker.

Similarly, T-cell lineage origin can be tracked by analysis of the T-cell receptor (TCR) gene rearrangement status. Thus, I performed a rearrangement PCR on the TCR $\beta$  locus, which revealed that CD4<sup>+</sup>/Gr1<sup>+</sup> cell are derived from the T-cell lineage. These



results indicate that while formation of B-cell leukemia is completely abolished by hypomethylation, it still allows occasional outgrowth of malignant T cells.



**Figure 19: Genomic rearrangement status at immunoglobulin and T-cell receptor loci**

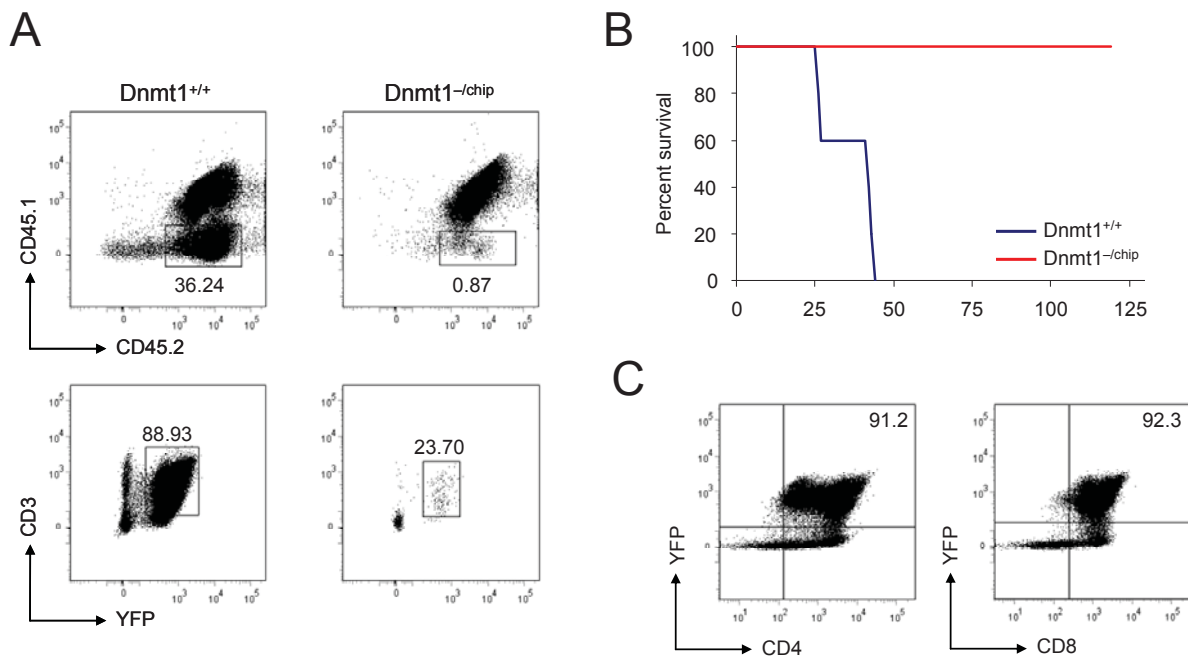
**A)** PCR on genomic DNA (gDNA) from B220<sup>+</sup>/Gr1<sup>+</sup> blasts sorted from *Dnmt1*<sup>+/+</sup> and *Dnmt1*<sup>-chip</sup> *Myc-Bcl2* leukemias. Spleen cells served as positive control for a rearranged locus; E86 fibroblast cell line gDNA served as control for germ line configuration. GL, germ line. Asterisks indicate products of different rearrangement combinations **B)** PCR on gDNA from sorted *Dnmt1*<sup>-chip</sup> CD4<sup>+</sup>/Gr1<sup>+</sup> cells to evaluate the rearrangement status of the T-cell receptor  $\beta$  locus. Thymocytes served as positive control, bone marrow cells (BM) served as germ line configuration control. GL, germ line.

Taken together, these results demonstrate that hypomethylation does not allow B-cell ALL development from a bi-potential leukemia initiating cell indicating that lineage fate choices in leukemia are determined by methylation levels.

### 3.1.4 Hypomethylation blocks T-ALL development

As T-ALL development was observed in rare cases of hypomethylated *Myc-Bcl2* leukemia, I tested whether hypomethylation was generally compatible with growth of T-lineage leukemias. Consequently, I chose a specific T-ALL leukemia model applying a constitutively active variant of *Notch1* as driving oncogene. In normal hematopoiesis, *Notch1* acts as a key regulator in the commitment of hematopoietic progenitors to the T-cell lineage [Aifantis, et al., 2008]. Constitutive *Notch1* signaling,

achieved by forced expression of the active, intracellular domain of *Notch1* (*Notch1-IC*) was shown to lead to T cell leukemia formation [Allman, et al., 2001; Pear, et al., 1996]. I tested whether *Notch1-IC* induced T-cell lymphomas could be given rise to by hypomethylated hematopoietic cells by retrovirally introducing *Notch1-IC* into *Dnmt1*<sup>-chip</sup> progenitor cells. Peripheral blood analysis 18 days post-transplantation revealed engraftment of both *Dnmt1*<sup>+/+</sup> and *Dnmt1*<sup>-chip</sup> *Notch1-IC* cells, however, presence of hypomorphic donor cells was markedly lower compared to wildtype counterparts. Cells expressing *Notch1-IC-YFP* were positive for CD3, indicating their T-lineage character (**Figure 20A**).



**Figure 20: Acute T-cell lymphoma induced by *Notch1-IC***

**A)** FACS analysis of peripheral blood 18 days after transplantation of mice reconstituted with either *Dnmt1*<sup>+/+</sup> or *Dnmt1*<sup>-chip</sup> cells transduced with *Notch1-IC*. Upper plots depict cells within donor gate (CD45.1<sup>-</sup>CD45.2<sup>+</sup>). Lower plots indicate percentages of *Notch1-IC-YFP*<sup>+</sup> cells. Data from one representative animal is shown. **B)** Survival curve of *Dnmt1*<sup>+/+</sup> or *Dnmt1*<sup>-chip</sup> *Notch1-IC* reconstituted mice. *Dnmt1*<sup>-chip</sup> *Notch1-IC* recipients did not show *Notch1-IC-YFP*<sup>+</sup> cells 120 days after transplantation and were consequently declared disease-free. n=5 for each genotype. **C)** FACS analysis of *Dnmt1*<sup>+/+</sup> leukemias displayed co-expression of CD4 and CD8 on YFP<sup>+</sup> cells indicative of T-ALL.

Whereas *Notch1-IC* wildtype control cells were able to induce leukemia in recipient mice, transduced hypomethylated cells were not able to initiate leukemia in recipient

animals (**Figure 20B**). *Dnmt1*<sup>+/+</sup> *Notch1-IC* leukemias displayed an immature T-cell phenotype marked by CD4<sup>+</sup>CD8<sup>+</sup> double-positive T-cells (**Figure 20C**).

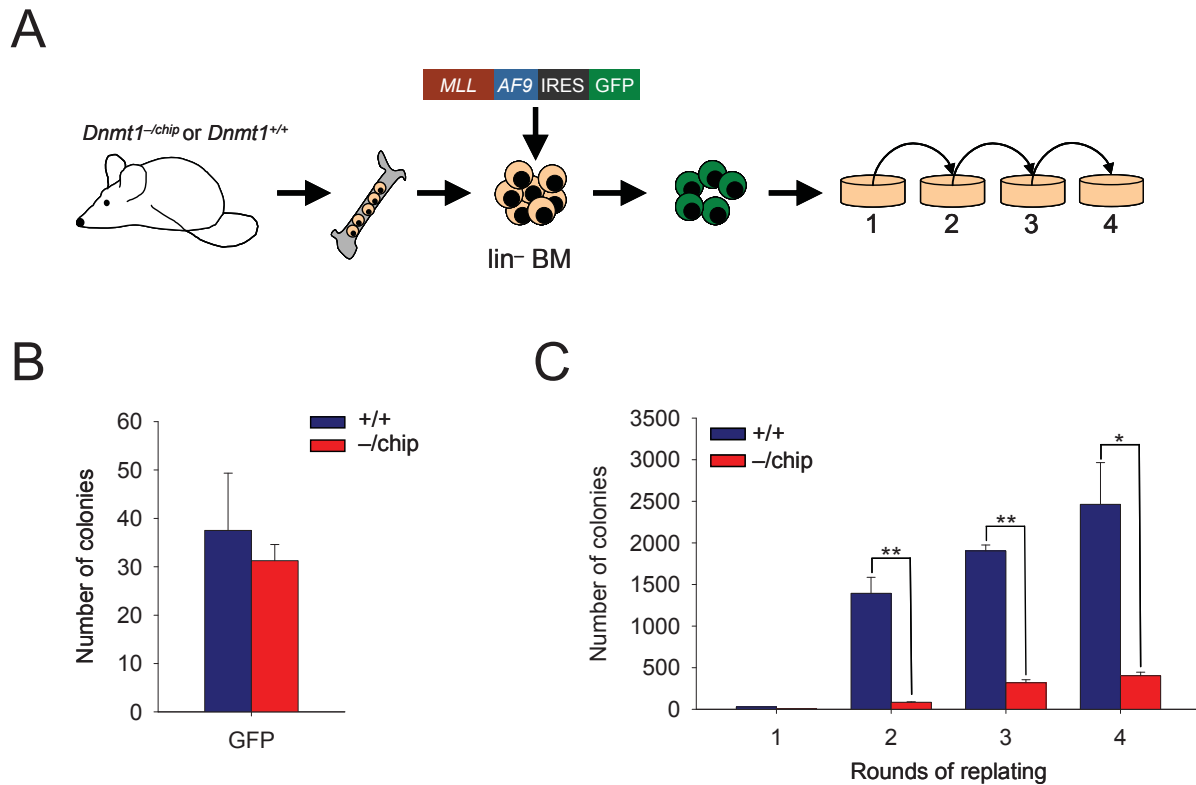
### 3.2 DNA methylation controls leukemia cell self-renewal

The studies presented in the previous section revealed that *Myc-Bcl2 Dnmt1*<sup>-/-chip</sup> leukemias developed with prolonged latencies compared to *Dnmt1*<sup>+/+</sup> leukemias. This observation suggested that leukemia stem cells (LSCs) rely on constitutive DNA methylation to perform proper self-renewal. However, *Myc-Bcl2 Dnmt1*<sup>-/-chip</sup> leukemias are devoid of the neoplastic B-cell population, which might also be causal for delayed leukemia onset. Consequently, in order to test the dependency of LSC self-renewal on constitutive DNA methylation, a leukemia model with a uniform, homogenously myeloid phenotype would be preferential. For this reason I chose the *MLL-AF9* fusion as oncogene, which induces an acute myeloid leukemia (AML) in mice [Corral, et al., 1996]. *MLL-AF9* is one of more than 60 fusion products of chromosomal translocations involving the human mixed lineage leukemia (*MLL*) gene, which are predominantly found in infant leukemia and occur in patients with AML and ALL as well as in a substantial proportion of biphenotypic mixed-lineage leukemias, [Dimartino and Cleary, 1999; Meyer, et al., 2009]. *MLL* fusion proteins display potent transcriptional activators. In *MLL*-mediated leukemogenesis *MLL*-target genes, which mostly belong to the transcription factor group of *Hox* (homeobox) genes, are constitutively activated inhibiting hematopoietic maturation and leading to leukemia [Slany, 2005].

#### 3.2.1 Reduced self-renewal of hypomethylated leukemia cells *in vitro*

*MLL-AF9* has frequently been used as an oncogene in leukemia research and it can be applied for transforming hematopoietic cells *in vitro*, providing cultured cells with unlimited proliferation capacities. Moreover, *MLL-AF9* can be used to induce AML in mice upon transplantation of transduced cells [Somervaille and Cleary, 2006]. In order to investigate the effect of DNA hypomethylation on leukemic self-renewal, I conducted an *in vitro* serial replating assay in semisolid methylcellulose. Cells growing in such semisolid medium form colonies originating from a single starting cell and in case of *MLL-AF9* transformed cells, it has been shown that cells expanded

from every colony have the capacity to induce a leukemia in mice upon their transplantation [Somerville and Cleary, 2006]. Hence, colony assays display a valuable tool to quantify self-renewal capacities by counting the number of colonies growing from a certain number of cells. In serial replating assays a specific cell number is seeded in methylcellulose and after several days of culture colony numbers are determined and a certain number of cells is re-seeded on the next plate. This can be applied for several rounds of replating given that there is colony growth during the previous round. Hematopoietic cells without transforming event possess very limited self-renewal capacities, which is rapidly 'out-diluted' during *in vitro* culture leading to decreasing colony formation in the course of replating rounds. On the contrary, if cells are transformed with an oncogene such as *MLL-AF9*, they acquire unlimited self-renewal causing increasing colony formation and allowing virtually infinite replating. Thus, *Dnmt1*<sup>-/chip</sup> and *Dnmt1*<sup>+/+</sup> cells were immortalized with a retroviral *MLL-AF9* fusion construct and a serial replating experiment was performed (**Figure 21A**). To test whether *Dnmt1*<sup>-/chip</sup> cells generally show the same response to growth factors in the culture medium as *Dnmt1*<sup>+/+</sup> cells, leading to equal colony numbers under non-transforming conditions, I assessed colony numbers of cells infected with a *MSCV-IRES-GFP* construct. As depicted in **Figure 21B** both *Dnmt1*<sup>-/chip</sup> *GFP*<sup>+</sup> and *Dnmt1*<sup>+/+</sup> *GFP*<sup>+</sup> cells gave rise to equal numbers of colonies suggesting that the overall colony-forming ability is not affected by DNA hypomethylation. In contrast to that, *Dnmt1*<sup>-/chip</sup> cells transduced with *MLL-AF9* showed a severe replating deficiency compared to their wildtype counterparts, indicating that they had impaired self-renewal capacity *in vitro* (**Figure 21C**).



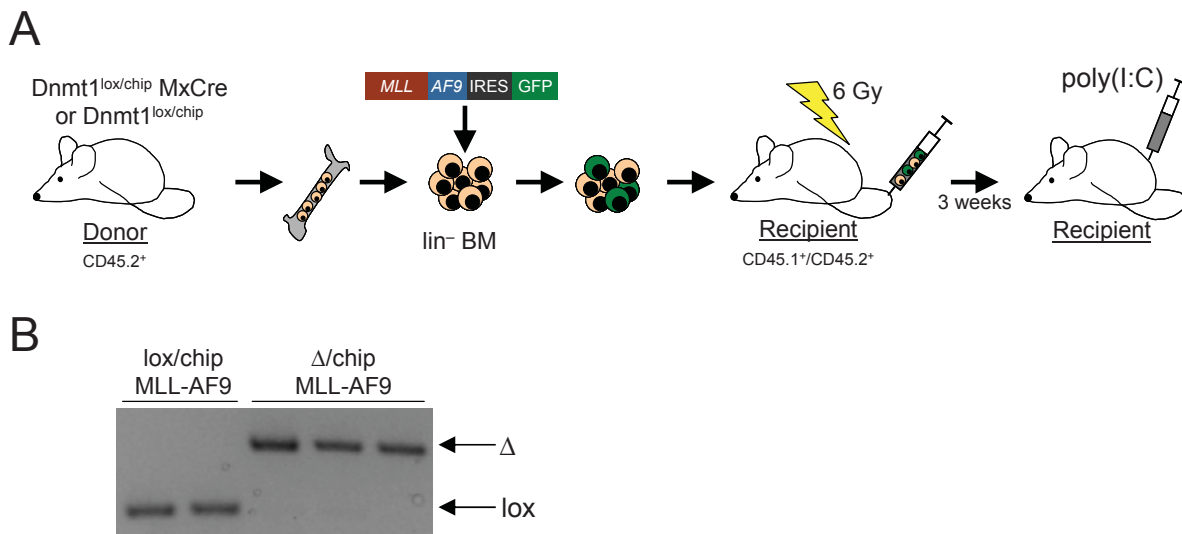
**Figure 21: Assessment of leukemic self-renewal by colony assays**

**A)** Experimental setup for *in vitro* colony assay of *MLL*-*AF9* transformed *Dnmt1*<sup>+/+</sup> and *Dnmt1*<sup>-chip</sup> bone marrow cells **B)** *Dnmt1*<sup>+/+</sup> and *Dnmt1*<sup>-chip</sup> bone marrow cells transduced with *GFP*-expressing control virus gave rise to equal numbers of colonies in methylcellulose. Experiment was done in triplicates; data show is mean  $\pm$  s.d. **C)** Serial replating assay of *MLL*-*AF9*-transduced *Dnmt1*<sup>+/+</sup> and *Dnmt1*<sup>-chip</sup> bone marrow cells. *GFP*<sup>+</sup> cells were sorted, and for each round, 5,000 cells were plated in methylcellulose. After 5 d, colonies were counted and cells were re-seeded on the next plate. Data show mean  $\pm$  s.d of one representative experiment. Experiment was done twice in triplicates.

### 3.2.2 Reduced self-renewal of hypomethylated leukemia cells *in vivo*

Having demonstrated that leukemic self-renewal is diminished due to hypomethylation *in vitro*, I investigated self-renewal potential of hypomethylated *MLL*-*AF9* leukemias *in vivo*. For this experiment an inducible *Dnmt1* knockdown mouse model termed *Dnmt1*<sup>lox/chip</sup> was applied (description see 2.2.2.1). Crossing these mice with *Mx1Cre* mice allows to induce Cre-expression and consequently *Dnmt1* knockdown by injection of Poly(I:C). The 'floxed' allele is termed *Dnmt1*<sup>lox</sup>, whereas the functional knockout allele which is created by Cre-excision is termed *Dnmt1*<sup>Δ</sup>. This conditional *Dnmt1* knockdown mouse model was chosen to avoid any experimental bias due to

unequal engraftment capacities of hypomethylated cells, as the *Dnmt1*<sup>lox</sup> allele is excised after transplantation and engraftment of cells (**Figure 22A**). To control the results obtained with *Dnmt1*<sup>lox/chip</sup> *MxCre* cells they were compared to *Dnmt1*<sup>lox/chip</sup> cells without the *MxCre* allele. Recipient mice of *MLL-AF9* transduced cells were injected with Poly(I:C) three weeks after transplantation to trigger *Dnmt1* excision in those cells carrying the *MxCre* allele and leukemia development was monitored by weekly blood analysis.



**Figure 22: MLL-AF9 leukemia with conditional *Dnmt1* knockdown cells**

**A)** Experimental setup: Lin<sup>-</sup> BM cells from *Dnmt1*<sup>lox/chip</sup> *MxCre* or *Dnmt1*<sup>lox/chip</sup> mice were infected with *MLL-AF9* retrovirus and transplanted into sublethally irradiated SJLx129ola recipients. Three weeks after transplantation, recipients were injected with Poly(I:C) every other day for a total of five injections.

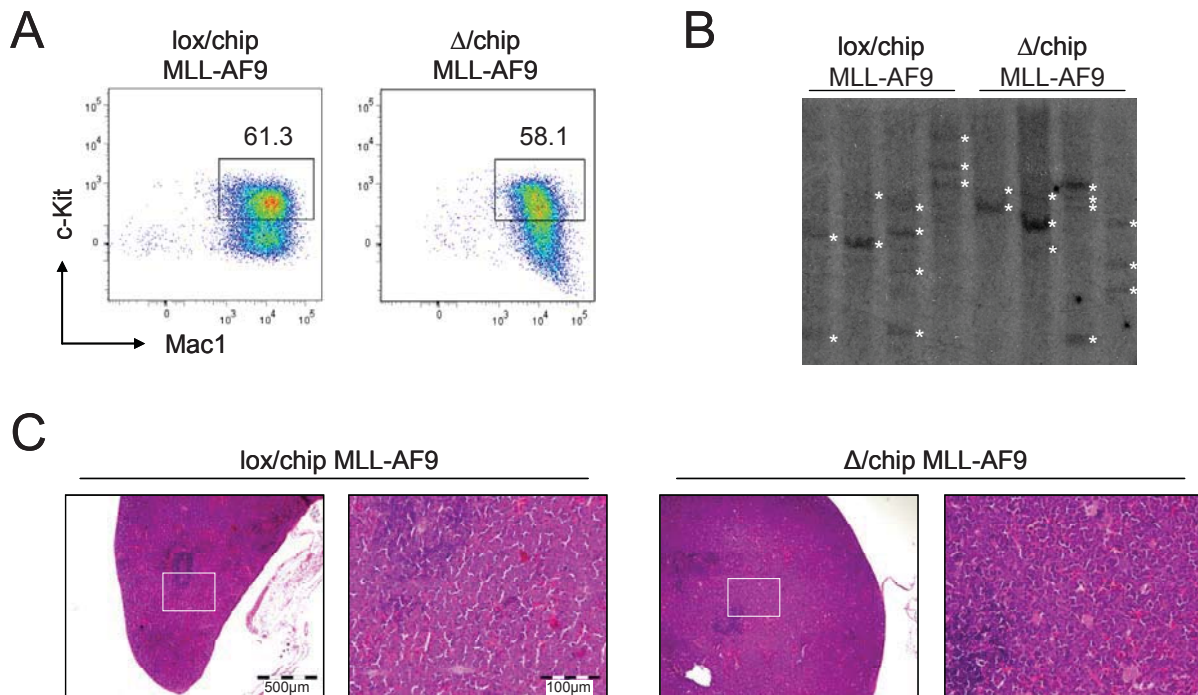
**B)** PCR on genomic DNA from *Dnmt1*<sup>Δ/chip</sup> *MLL-AF9* and *Dnmt1*<sup>lox/chip</sup> *MLL-AF9* leukemia cells confirms near-complete excision of the *Dnmt1*<sup>lox</sup> allele.

Similar to the *Myc-Bcl2* model, *Dnmt1*<sup>Δ/chip</sup> *MLL-AF9* cell transplantation led to leukemia with prolonged latency with a median survival after Poly(I:C) of 48.2 ± 11.8 days (*Dnmt1*<sup>lox/chip</sup> *MLL-AF9*) and 61.95 ± 13.5 days (*Dnmt1*<sup>Δ/chip</sup> *MLL-AF9*), respectively ( $P = 0.0005$ ). The cells of *Dnmt1*<sup>Δ/chip</sup> *MLL-AF9* leukemias displayed near-complete excision of the *Dnmt1*<sup>lox</sup> allele (**Figure 22B**).

Furthermore the leukemias were characterized concerning their clonality, the cells' surface marker phenotype and the histological status of the infiltrated organs (**Figure 23A-C**). Leukemic cells found in bone marrow, spleen, thymus, blood and lymph



nodes were all positive for the myeloid marker Mac1. The majority of blast cells co-expressed the 'progenitor marker' c-Kit, a marker which is usually, i.e. in a physiological situation, not found on myeloid cells outside of the bone marrow.



**Figure 23: Characterization of *MLL-AF9* leukemias**

**A)** Representative FACS plots of GFP<sup>+</sup> splenocytes of *Dnmt1*<sup>lox/chip</sup> *MLL-AF9* and *Dnmt1* <sup>$\Delta$ /chip</sup> *MLL-AF9* diseased animals. **B)** Southern blot analysis of genomic DNA isolated from spleens of each four *lox/chip* and  $\Delta$ /*chip* *MLL-AF9* leukemic mice using a GFP-specific probe showed a comparable number of retroviral integration sites. **C)** Histological analysis of infiltrated spleens with hematoxylin and eosin staining. The right pictures show the magnification of the area indicated by the white box in the left pictures, respectively. Sizes are indicated by the measure in the pictures. Histological analyses were performed by Marco Prinz (University of Freiburg).

To analyze the clonality status of the leukemias, genomic DNA from leukemic cells was digested with two different enzymes, with one of them cutting within the inserted retroviral sequence and the other one not cutting within this area. Subsequent southern blotting and hybridization with a GFP-specific probe demonstrated that both *lox/chip* and  $\Delta$ /*chip* leukemias were oligoclonal with comparable numbers of integration sites (**Figure 23B**). Histological analysis of leukemic spleens, which was done in cooperation with Marco Prinz (University of Freiburg) showed high infiltration of leukemia cells causing damage and disappearance of physiological lymph follicle

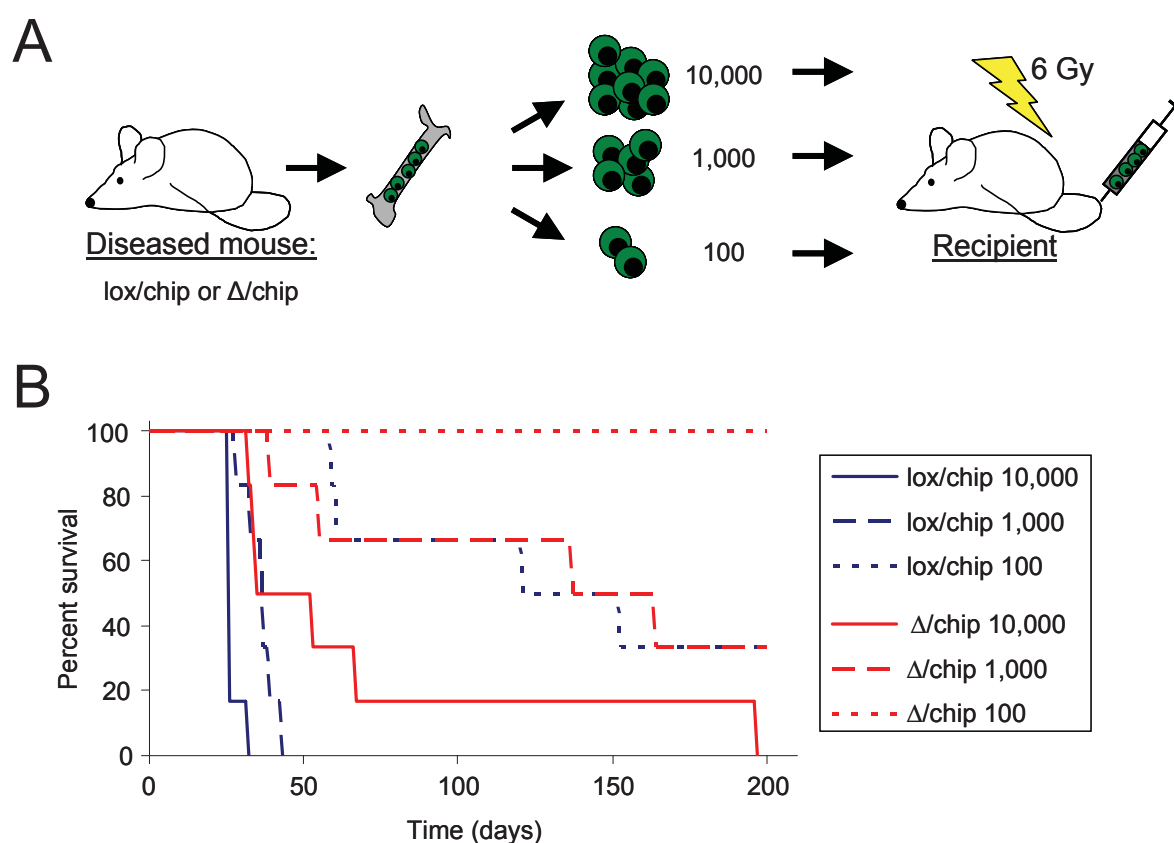
structures (**Figure 23C**).

These data indicate an impaired development of *Dnmt1*<sup>Δ/chip</sup> leukemias causing prolongation of disease latency. The phenotype of the emerging leukemias, however, is the same.

### 3.2.3 Hypomethylation causes reduction of functional LSCs

The results presented in the previous section suggested decreased self-renewal abilities of hypomethylated leukemia cells, however, the impairment of cells with self-renewal potential, the LSCs, was not quantified. To this end, I performed a limiting dilution experiment, which allows to determine the number of cells possessing LSC function within a given leukemia. This experiment is based on the fact that LSCs are, depending on the type of leukemia, not precisely or not at all phenotypically characterized and thus not identifiable. However, LSCs are defined by their function, which is that they are able to initiate a leukemia from a single cell level. According to this definition, the transplantation of a single LSC should be able to give rise to leukemia in the recipient. In a limiting dilution experiment, decreasing numbers of leukemia cells are transplanted into recipient mice and the proportion of recipients developing leukemia allows to determine the number of functional LSCs in the donor population. The experimental setup is illustrated in **Figure 24A** and the results from the experiment are summarized in **Table 2**. The obtained results allow to calculate the frequency of functional LSCs within the leukemic cell population: An LSC frequency of 1 in 91 *Dnmt1*<sup>lox/chip</sup> MLL-AF9 cells and 1 in 1,072 *Dnmt1*<sup>Δ/chip</sup> MLL-AF9 cells was determined for the respective leukemias. Additionally to this clearly reduced LSC frequency, diminished stem cell function is also visible from the drastic latency differences in the low cell number transplantations (**Figure 24B**).





**Figure 24: Limiting dilution experiment**

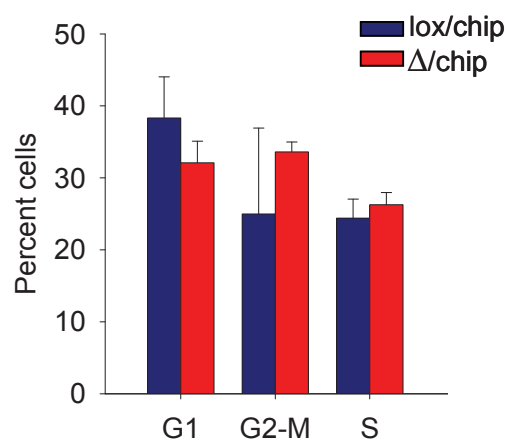
**A)** Experimental setup of limiting dilution assay: 10,000, 1,000 or 100  $Dnmt1^{lox/chip}$  MLL-AF9 and  $Dnmt1^{\Delta/chip}$  MLL-AF9 leukemic cells were transplanted into sublethally irradiated secondary recipients.

**B).** Survival of secondary recipient mice injected with limiting dilutions of lox/chip or  $\Delta$ /chip MLL-AF9 bone marrow cells (containing >95% GFP<sup>+</sup> leukemic cells).

**Table 2: Occurrence of leukemia in recipients transplanted with MLL-AF9 leukemia cells in limiting dilution experiment**

	$Dnmt1^{lox/chip}$ MLL-AF9			$Dnmt1^{\Delta/chip}$ MLL-AF9		
Transplanted cells (#)	Transplanted mice (#)	Leukemic mice (#)	Median latency (days)	Transplanted mice (#)	Leukemic mice (#)	Median latency (days)
10,000	6	6	26	6	6	69
1,000	6	6	35	6	4	98
100	6	4	98	6	0	-

Given the huge discrepancy in the aggressiveness of *Dnmt1* <sup>$\Delta$ /chip</sup> leukemias compared to *Dnmt1*<sup>lox/chip</sup> leukemias, it was further investigated whether this is exclusively based on reduced functioning of LSCs or whether the whole leukemia bulk is affected by hypomethylation. To assess this,  $\Delta$ /chip leukemic blasts as well as the control cells were cultured *in vitro*. After several passages, cell cycle profiles of harvested cells were determined by propidium iodide FACS analysis. This revealed an equal distribution of cell cycle phases and consequently similar proliferation rates (Figure 25).



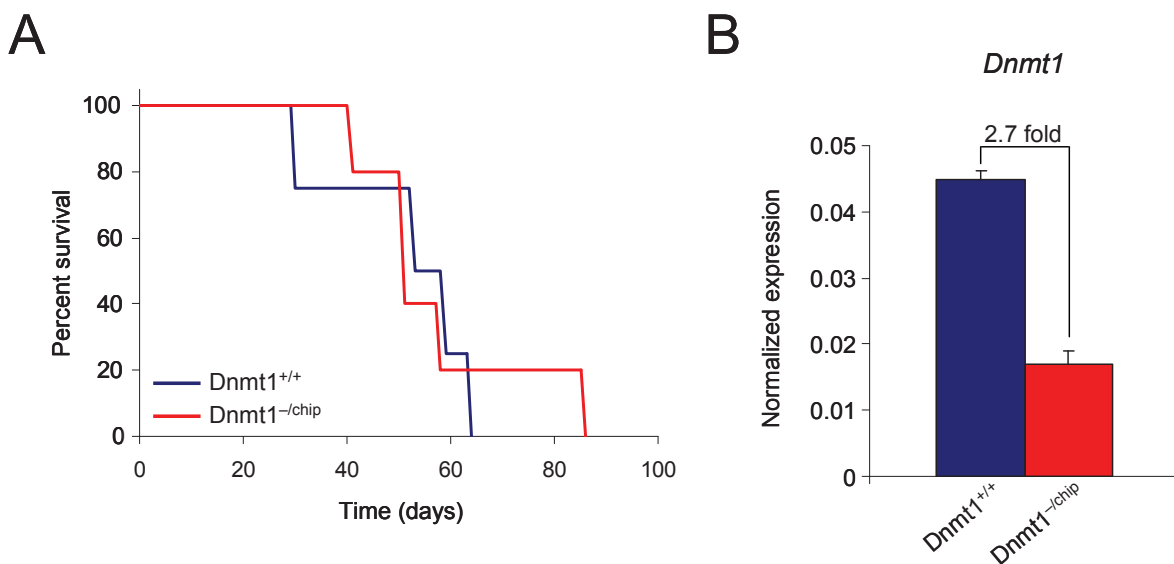
**Figure 25: Proliferation of MLL-AF9 leukemia bulk cells**

*Dnmt1*<sup>lox/chip</sup> and *Dnmt1* <sup>$\Delta$ /chip</sup> MLL-AF9 blasts were cultivated *in vitro* for three weeks in liquid culture with growth conditions for myeloid cells (IL3, IL6 and SCF). Harvested cells were ethanol-fixed, stained with propidium iodide and analyzed by FACS. Apoptotic cells and doublets were gated out.

### 3.2.4 Leukemogenesis is unaffected by hypomethylated stroma

The results presented in the previous chapters revealed that hypomethylation of leukemic cells strongly affects their function both concerning their lineage differentiation potential and their stem cell self-renewal. These effects were clearly cell intrinsic as in the chosen experimental setup hypomethylated leukemias were generated in wildtype recipient mice. Consequently, any effects due to hypomethylation result from the transplanted leukemia cells, with stroma cells of the recipient mouse being normally methylated. In order to test whether the methylation status of the recipients' bone marrow niche is critical for leukemia development, I generated wildtype (*Dnmt1*<sup>+/+</sup>) MLL-AF9 induced leukemic mice. Cells isolated from

these mice were subsequently transplanted into recipients providing either hypomethylated (*Dnmt1*<sup>-/-chip</sup>) or normally methylated (*Dnmt1*<sup>+/+</sup>) bone marrow microenvironment. To avoid any experimental bias from cell-to-cell interactions between leukemia cells and differentially methylated recipient cells, recipient bone marrow cells were removed by lethal irradiation. Recipients received *Dnmt1*<sup>+/+</sup> bone marrow support alongside the leukemic cells creating a leukemia model with *Dnmt1*<sup>+/+</sup> leukemia and hematopoietic system in a hypomethylated niche. **Figure 26A** illustrates that leukemias developed with similar latencies in *Dnmt1*<sup>+/+</sup> and *Dnmt1*<sup>-/-chip</sup> recipients. *Dnmt1*<sup>+/+</sup> recipients succumbed to leukemia with a median latency of 50.5 ± 15.0 days and *Dnmt1*<sup>-/-chip</sup> recipients developed leukemia after 56.4 ± 17.1 days (P = 0.60).



**Figure 26: Leukemia development in hypomethylated microenvironment**

**A)** 5000 *Dnmt1*<sup>+/+</sup> MLL-AF9 leukemia cells were transplanted into lethally irradiated (10.5 Gy) *Dnmt1*<sup>-/-chip</sup> or *Dnmt1*<sup>+/+</sup> recipients. 10<sup>6</sup> *Dnmt1*<sup>+/+</sup> bone marrow cells were transplanted as support alongside to provide radioprotection. Survival curve depicts leukemia development of 4 (*Dnmt1*<sup>+/+</sup>) or 5 (*Dnmt1*<sup>-/-chip</sup>) recipients respectively. **B)** Real time RT-PCR of *Dnmt1*<sup>+/+</sup> and *Dnmt1*<sup>-/-chip</sup> bone marrow stroma cells confirmed down-regulation of *Dnmt1* expression. Data were normalized to the expression of  $\beta$ -actin. Results represent the mean ± s.d. of two independent experiments.

Furthermore, the leukemia phenotype was not influenced by the genotype of the recipient (data not shown). *Dnmt1* knockdown in *Dnmt1*<sup>-/-chip</sup> stroma cells was

successfully confirmed as shown in **Figure 26B**. These data suggest that, in contrast to the striking cell intrinsic effects of hypomethylation on leukemia development, the methylation status of the bone marrow stroma does not affect leukemic growth or phenotype.

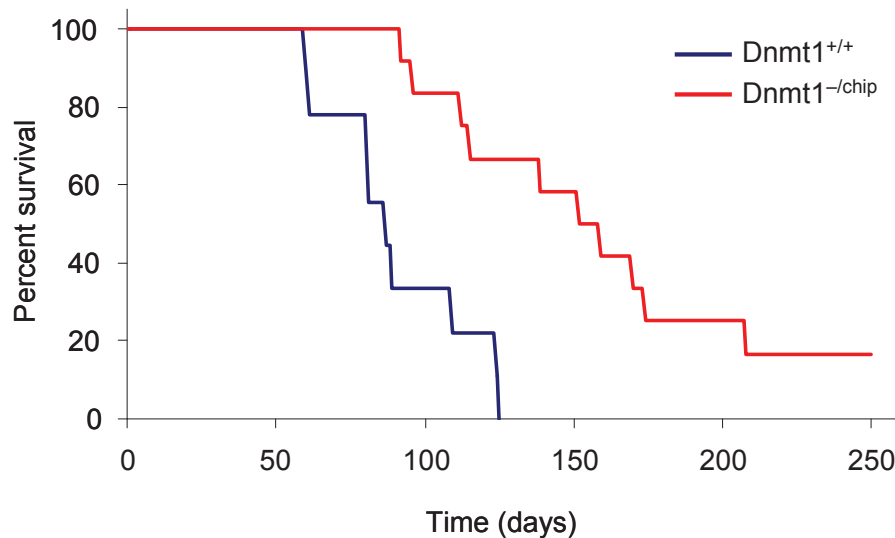
### 3.3 Hypomethylated LSCs display impaired self-renewal

The data presented in chapter 3.2 suggested that hypomethylation strongly interferes with LSC function. However, in all experiments stem cell function was tested in the context of the bulk leukemia. In case of *MLL-AF9* driven leukemia in mice, several studies have been dedicated to phenotypically describe the LSC. Scott Armstrong's group found a highly enriched incidence of LSCs in a subpopulation of *MLL-AF9* leukemia cells, which featured a cell surface marker composition (IL-7R<sup>-</sup>Lin<sup>-</sup>Sca-1<sup>-</sup>c-Kit<sup>high</sup>CD34<sup>+</sup>FcγRII/III<sup>+</sup>) usually found on granulocyte-monocyte progenitors (GMPs). The accordingly termed GMP-like leukemic cells (L-GMP) were found to contain leukemia-initiating cells with a frequency of approximately 1 in 6 cells [Krivtsov, et al., 2006]. Further studies found LSC activity to be enriched in the Lin<sup>-</sup>c-Kit<sup>+</sup> compartment of *MLL-AF9* leukemia cells [Somervaille and Cleary, 2006; Somervaille, et al., 2009]. In conclusion of these two findings, c-Kit appears to be one of the most critical markers defining cells with LSC function in *MLL-AF9* driven leukemias.

#### 3.3.1 Generation of *Dnmt1*<sup>-chip</sup> *MLL-AF9* leukemias

In order to more thoroughly investigate effects of hypomethylation on LSC function, I purified cells highly expressing c-Kit to test their self-renewal abilities both *in vitro* and *in vivo*. The inducible *Dnmt1*-knockdown model applied in the previously described experiments turned out to be sub-optimal for studies involving repeated rounds of transplantation, as, over time, cells which had escaped *Dnmt1*-excision and consequently displayed a growth advantage due to a wildtype-like methylation status, overgrew the excised, hypomethylated cells (data not shown). For this reason I generated leukemias originating from *MLL-AF9* transduced *Dnmt1*<sup>-chip</sup> bone marrow progenitors or their wildtype counterparts (*Dnmt1*<sup>+/+</sup>), respectively. To this end *Dnmt1*<sup>-chip</sup> and *Dnmt1*<sup>+/+</sup> cells were retrovirally transduced with *MLL-AF9* and transplanted into recipient animals. All but two *Dnmt1*<sup>-chip</sup> *MLL-AF9* recipients

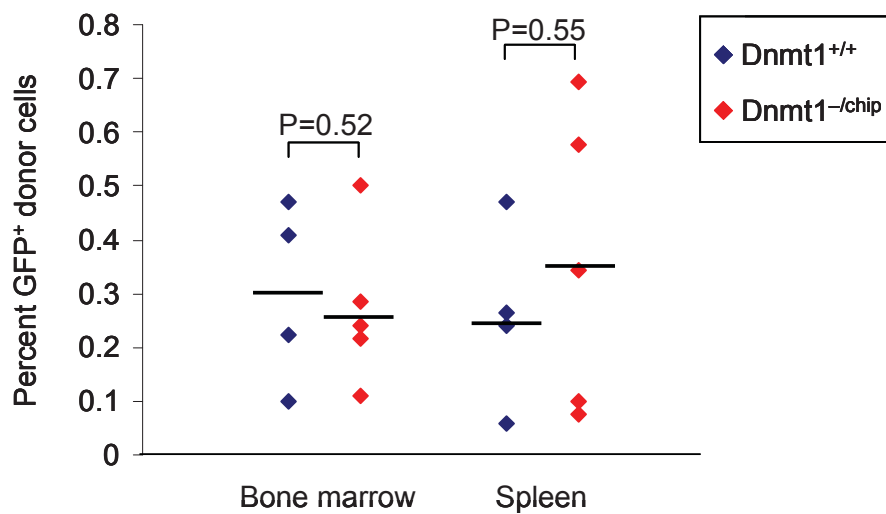
developed AML, however, mice which developed leukemia displayed significantly increased disease latencies of  $140.8 \pm 37.0$  days compared to the wildtype counterparts, which succumbed to AML after a median latency of  $89.8 \pm 24.1$  days post-transplantation ( $P = 0.003$ ,  $n = 9-12$ ); (**Figure 27**).



**Figure 27: *Dnmt1*<sup>-/chip</sup> and *Dnmt1*<sup>+/+</sup> MLL-AF9 leukemia development**

Survival curve depicts leukemia development of 9 (*Dnmt1*<sup>+/+</sup> MLL-AF9) or 12 (*Dnmt1*<sup>-/chip</sup> MLL-AF9) recipients respectively. Two recipients of *Dnmt1*<sup>-/chip</sup> MLL-AF9 were devoid of GFP<sup>+</sup> cells after 250 days post-transplantation and were consequently considered as disease-free.

To exclude engraftment deficiencies of *Dnmt1*<sup>-/chip</sup> MLL-AF9 cells to be responsible for prolonged disease latencies, I performed a short-term engraftment assay, in which *Dnmt1*<sup>-/chip</sup> and *Dnmt1*<sup>+/+</sup> leukemia cells were tested for their ‘homing’ capacity to the recipients’ hematopoietic organs following intravenous injection. Analysis of bone marrow and spleen of recipient animals 20 hours after transplantation revealed an equal contribution of *Dnmt1*<sup>-/chip</sup> and *Dnmt1*<sup>+/+</sup> leukemic cells in these organs and consequently comparable engraftment abilities (**Figure 28**).



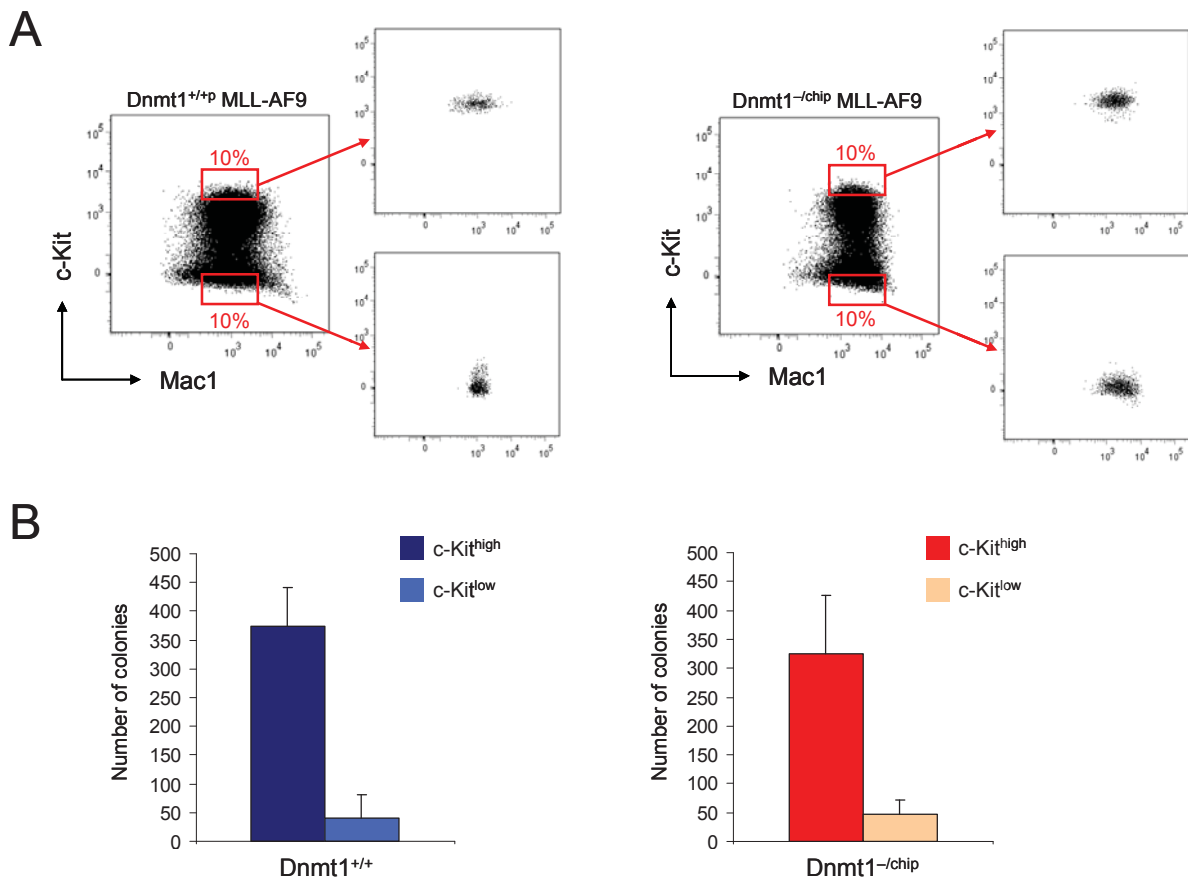
**Figure 28: *MLL*-AF9 leukemic blast homing**

$10^7$  *Dnmt1*<sup>-chip</sup> or *Dnmt1*<sup>+/+</sup> unsorted leukemia cells were transplanted into sublethally irradiated SJLx129ola mice. Recipients were sacrificed 20 hours post-transplantation. Each square box in the plot represents the mean percentage of GFP<sup>+</sup> donor cells in bone marrow (left) and spleen (right) of three recipients injected with leukemic cells from one donor. 4 *Dnmt1*<sup>+/+</sup> *MLL*-AF9 and 5 *Dnmt1*<sup>-chip</sup> *MLL*-AF9 leukemias were analyzed for their short-term engraftment ability. The black bars indicate the mean percentage of GFP<sup>+</sup> cells of all 4 or 5 transplantations, respectively. Both bone marrow ( $P = 0.52$ ) and spleen ( $P = 0.55$ ) displayed no statistically significant differences in short-term homing capacity.

### 3.3.2 Hypomethylated LSCs display impaired self-renewal

Having successfully generated *Dnmt1*<sup>-chip</sup> and *Dnmt1*<sup>+/+</sup> *MLL*-AF9 leukemias, a cell population enriched for LSCs was purified from the leukemia bulk. To this end the c-Kit highest expressing leukemic cells, further on termed as c-Kit<sup>high</sup>, were FACS-sorted as illustrated in **Figure 29A**.

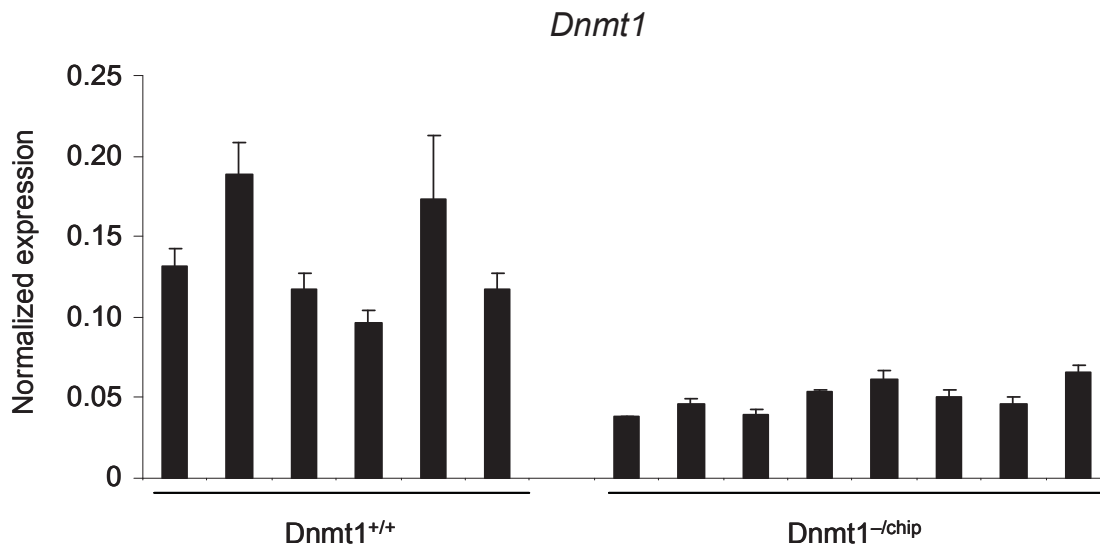
To ensure that *Dnmt1*<sup>-chip</sup> *MLL*-AF9 LSCs are harbored within the c-Kit<sup>high</sup> compartment, the c-Kit lowest expressing leukemia cells (c-Kit<sup>low</sup>) were sorted for comparison. Both c-Kit<sup>high</sup> and c-Kit<sup>low</sup> cells were plated in methylcellulose and colony numbers scored after 5 days of culture revealed consistently more colonies growing from c-Kit<sup>high</sup> cells. Cultured c-Kit<sup>high</sup> cells yielded 7.5 (*Dnmt1*<sup>+/+</sup>) or 5.5 (*Dnmt1*<sup>-chip</sup>) times more colonies than c-Kit<sup>low</sup> cells of the respective genotype. These data confirmed that LSCs are enriched in the c-Kit<sup>high</sup> cell population both in hypomethylated as well as in normally methylated leukemias (**Figure 29B**).



**Figure 29: Purification and testing of *MLL-AF9* LSCs**

**A)** Scheme for FACS sort purification of *Dnmt1*<sup>+/+</sup> and *Dnmt1*<sup>-/-chip</sup> *MLL-AF9* LSC enriched population. c-Kit<sup>high</sup> cells were sorted as the 10% c-Kit highest expressing cells and for comparison the 10% c-Kit lowest expressing cells were sorted (c-Kit<sup>low</sup>). FACS plots are gated on GFP<sup>+</sup> donor cells. Red boxes indicate the sorting gates. Purified populations are depicted in the right respective plots. **B)** 500 Sorted *Dnmt1*<sup>+/+</sup> and *Dnmt1*<sup>-/-chip</sup> c-Kit<sup>high</sup> and c-Kit<sup>low</sup> cells were plated in methylcellulose in the presence of IL3, IL6 and SCF and colonies were scored after 5 days. Values are mean ± s.d. number of colonies from 4 leukemias per genotype.

Furthermore, I tested *Dnmt1* expression in sorted c-Kit<sup>high</sup> cells to ensure that hypomethylation also occurs in this specific cell population. For this purpose, c-Kit<sup>high</sup> cells from several individual leukemias were individually tested for *Dnmt1* expression by real-time RT-PCR, the results of which are depicted in **Figure 31**. *Dnmt1* was found to be consistently down regulated in all tested *Dnmt1*<sup>-/-chip</sup> samples. On average, *Dnmt1* expression was reduced to 35.4% of wildtype *Dnmt1* level. Similarly, *Dnmt1* expression was decreased in *Dnmt1*<sup>-/-chip</sup> c-Kit<sup>low</sup> cells, which displayed 41.1% expression of wildtype *Dnmt1* (data not shown).

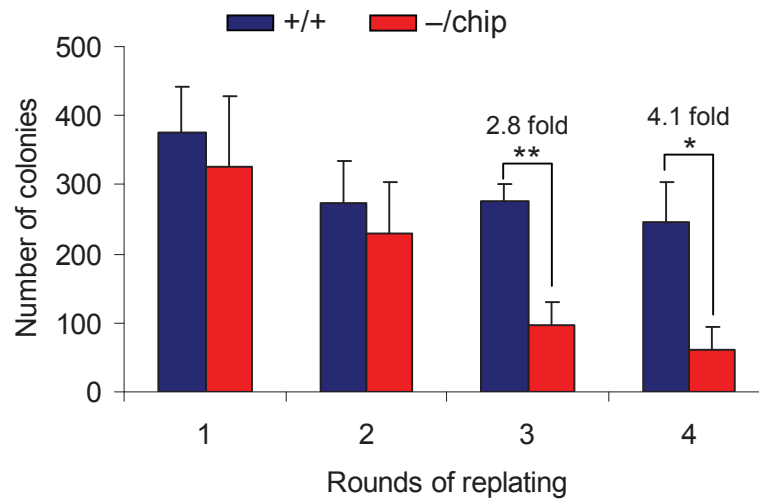


**Figure 30: Reduced *Dnmt1* expression in *Dnmt1*<sup>-/chip</sup> MLL-AF9 LSCs**

*Dnmt1* real-time RT-PCR on FACS sort purified *Dnmt1*<sup>+/+</sup> and *Dnmt1*<sup>-/chip</sup> MLL-AF9 c-Kit<sup>high</sup> cells. Cells from 6 or 8 individual leukemias were tested, respectively. Values are mean  $\pm$  s.d. of technical replicates.

To investigate self-renewal potential of LSC enriched leukemia cell populations, c-Kit<sup>high</sup> cells were sorted from *Dnmt1*<sup>+/+</sup> and *Dnmt1*<sup>-/chip</sup> MLL-AF9 leukemias, cultured in methylcellulose and subsequently replated as described before. *Dnmt1*<sup>+/+</sup> MLL-AF9 colony numbers slightly decreased in the second round but in the subsequent rounds *Dnmt1*<sup>+/+</sup> LSCs stably replated with constant colony numbers (**Figure 31A**). In contrast, *Dnmt1*<sup>-/chip</sup> LSCs yielded continuously decreasing colony numbers over four replating rounds (**Figure 31B**). These data suggest a progressive loss of self-renewal capacity in *Dnmt1*<sup>-/chip</sup> c-Kit<sup>high</sup> populations, whereas leukemic self-renewal is maintained in case of wildtype LSCs.

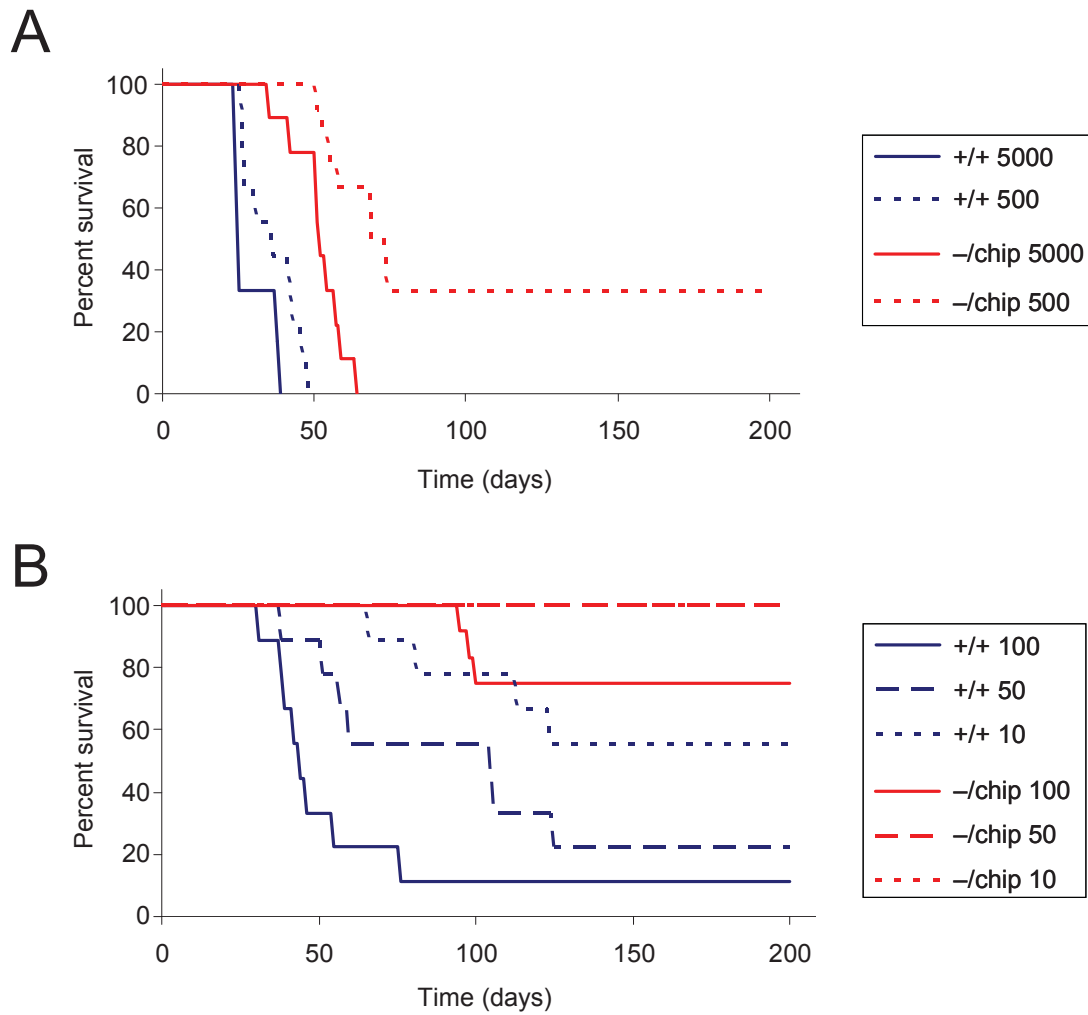




**Figure 31: Serial replating of *MLL-AF9* LSCs**

Bone marrow cells were isolated from leukemic *Dnmt1*<sup>+/+</sup> and *Dnmt1*<sup>-/chip</sup> *MLL-AF9* mice. 500 FACS sorted c-Kit<sup>high</sup> cells were plated in methylcellulose and replated after 5 days for a total of 4 rounds. Graphs illustrate colony numbers of *Dnmt1*<sup>+/+</sup> and *Dnmt1*<sup>-/chip</sup> *MLL-AF9* LSC replating assays. For each genotype c-Kit<sup>high</sup> cells from 4 individual leukemic animals were used. Values are mean  $\pm$  s.d..

To confirm the results gathered in the *in vitro* replating assays in an *in vivo* approach a limiting dilution assay with purified LSCs (c-Kit<sup>high</sup> cells) was conducted as described in section 3.2.2. The results of this limiting dilution experiment are summarized in **Table 3** and survival curves of recipient mice are depicted in **Figure 32**. Based on these numbers an LSC frequency of 1 in 33 was calculated for *Dnmt1*<sup>+/+</sup> *MLL-AF9* c-Kit<sup>high</sup> cells. In *Dnmt1*<sup>-/chip</sup> *MLL-AF9* c-Kit<sup>high</sup> cells, however, the determined LSC frequency was 1 in 478, consequently representing an 14.5 fold decreased frequency of functional LSCs. As observed in all previously described transplantations, development of hypomethylated leukemias took place with drastically prolonged latencies, presenting further prove for diminished leukemic potential of hypomethylated LSCs.



**Figure 32: Limiting dilution experiment with sorted c-Kit<sup>high</sup> leukemia cells**

Sublethally irradiated recipient mice were transplanted with 20,000 (only *Dnmt1*<sup>-chip</sup>, not depicted here), 5,000, 500, 100, 50 or 10 *Dnmt1*<sup>+/+</sup> or *Dnmt1*<sup>-chip</sup> FACS sorted c-Kit<sup>high</sup> *MLL-AF9* leukemic cells. Survival curves of recipient mice of 5,000 and 500 cells (**A**) and 100, 50 and 10 cells (**B**) are depicted. Mice which did not display GFP<sup>+</sup> cells after 200 days were considered as disease-free.

**Table 3: Occurrence of leukemia in recipients transplanted with FACS sorted c-Kit<sup>high</sup> MLL-AF9 leukemia cells in limiting dilution experiment**

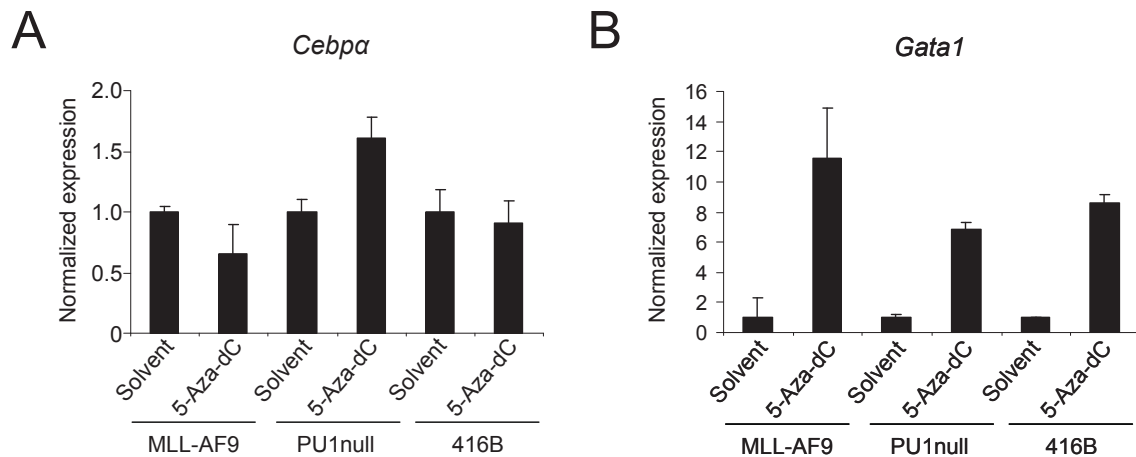
	<i>Dnmt1</i> <sup>+/+p</sup> MLL-AF9			<i>Dnmt1</i> <sup>-chip</sup> MLL-AF9		
Transplanted cells (#)	Transplanted mice (#)	Leukemic mice (#)	Median latency (days)	Transplanted mice (#)	Leukemic mice (#)	Median latency (days)
20,000	-	-	-	4	4	37.8
5,000	6	6	28.2	9	9	50.7
500	9	9	35.2	12	8	63.0
100	9	8	45.4	12	3	96.7
50	9	7	76.4	10	0	-
10	9	4	92.0	6	0	-

### 3.4 Hypomethylation induces expression of differentiation genes

#### 3.4.1 Pharmacological demethylation activates differentiation factors

In our previous studies of hypomethylation effects on hematopoietic stem cell (HSC) fates, we found that impaired HSC function in *Dnmt1*<sup>-chip</sup> mice is caused by premature expression of differentiation factors in these cells. In microarray based gene expression analysis, we found that transcriptomes of *Dnmt1*<sup>-chip</sup> HSCs were more closely related to myeloid progenitors compared to their wildtype counterparts. Several myeloerythroid specific factors were found to be increasingly expressed in the *Dnmt1*<sup>-chip</sup> HSC leading to partial differentiation of these cells causing diminished stem cell activity [Broske, et al., 2009]. Based on these findings activation of myeloerythroid genes caused by demethylation might display a mechanism by which hypomethylation can generally inhibit the renewal of cancer cells. To test this hypothesis, I treated different murine myeloid leukemia lines and primary myeloid

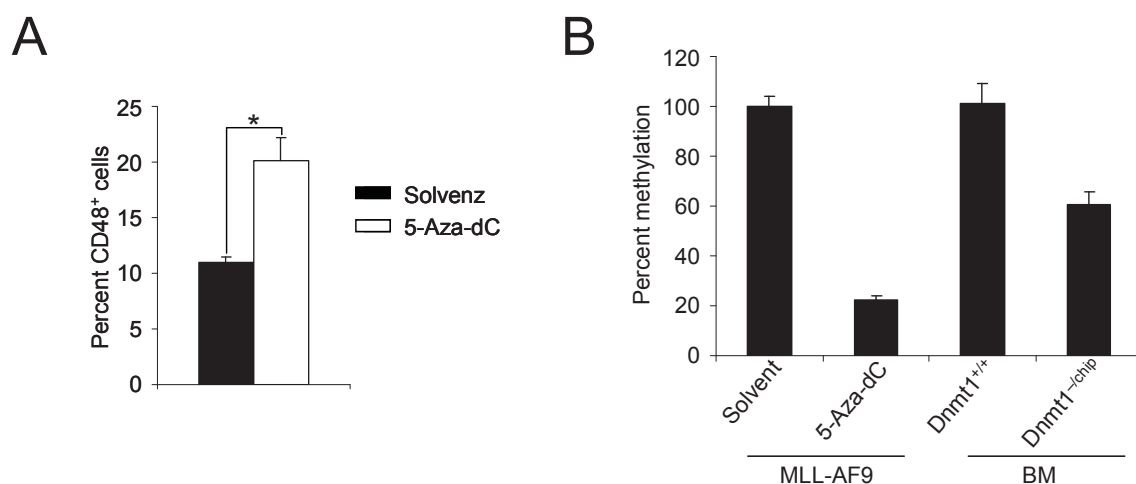
leukemia cells with 5-aza-2'-deoxycytidine (5-Aza-dC). After treatment, expression of *Gata1* and *Cebpa* on mRNA level and expression of the cell surface marker *CD48* was measured by FACS analysis. Whereas *Cebpa* expression remained unchanged, *Gata1* and expression was induced by 5-Aza-dC (**Figure 33A and B**).



**Figure 33: Activation of differentiation factors after demethylation I**

**A)** Real time RT-PCR of *Cebpa* and *Gata1* in two myeloid cell lines, 416B and PU1null, and primary leukemia cells derived from a moribund *MLL-AF9 Dnmt1<sup>+/-</sup>* mouse after 72 hours of treatment with 5-Aza-dC. Data were normalized to the expression of *Actb* and values of solvent samples were set to the value of 1. The experiment was done three times independently, with similar outcomes. Values are mean  $\pm$  s.d. **B)** FACS analysis showing enhanced expression of CD48 in 416B cells after 72 hour treatment with 5  $\mu$ M 5-Aza-dC. Data show mean  $\pm$  s.d. \* $P \leq 0.05$ .

Furthermore, CD48 expression was found to be up-regulated on 416B cells following 5-Aza-dC treatment (**Figure 34A**). Global genomic demethylation by 5-Aza-dC treatment was confirmed at the example of MLL-AF9 blasts (**Figure 34B**).

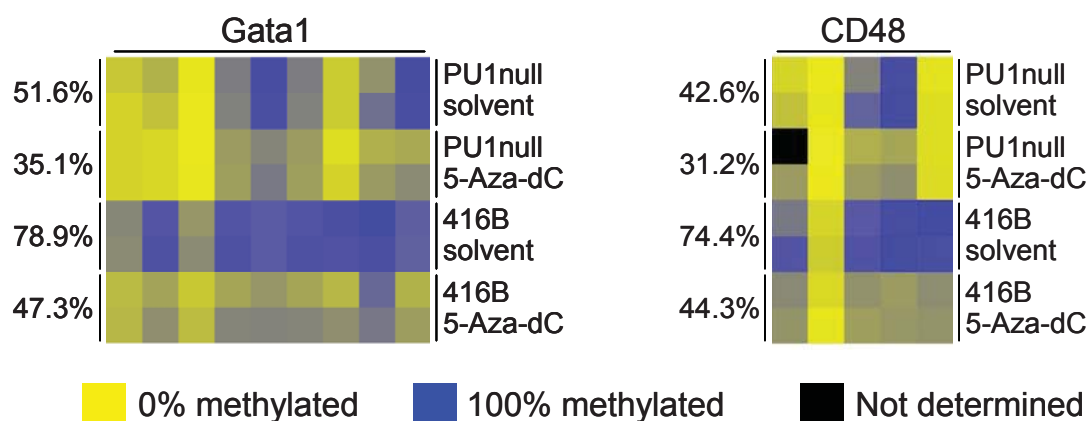


**Figure 34: Activation of differentiation factors after demethylation II**

**A)** FACS analysis showing enhanced expression of CD48 in 416B cells after 72 hour treatment with 5-Aza-dC. Data show mean  $\pm$  s.d. \* $P \leq 0.05$ . **B)** Global demethylation of 5-Aza-dC treated *Dnmt1*<sup>+/+</sup> MLL-AF9 blasts was shown using the Imprint Methylated DNA Quantification Kit. For comparison bone marrow (BM) cells of *Dnmt1*<sup>+/+</sup> and *Dnmt1*<sup>-chip</sup> mice is shown. Methylation of solvent treated MLL-AF9 blasts was set to 100%. Data show mean  $\pm$  s.d. of technical replicates.

### 3.4.2 5-Aza-dC treatment causes demethylation of *Gata1* and *Cd48* promoters

To test whether the observed activation of *Gata1* and *CD48* expression upon 5-Aza-dC treatment was caused by impaired silencing of the respective promoter and upstream regions the CpG methylation status of the treated cell lines was investigated. For this purpose, a MassARRAY was performed. This method allows to determine the CpG methylation status within a defined sequence. Thus, promoter sequences of *Gata1* and *Cd48* were analyzed applying MassARRAY technology, which was performed in cooperation with Sequenom, Hamburg, Germany. As illustrated in **Figure 35**, DNA methylation marks were partially removed from *Gata1* and *Cd48* promoter regions. These results suggest that *Gata1* and *Cd48* activation induced by pharmacological demethylation was caused by inhibition of DNA methylation mediated gene silencing.



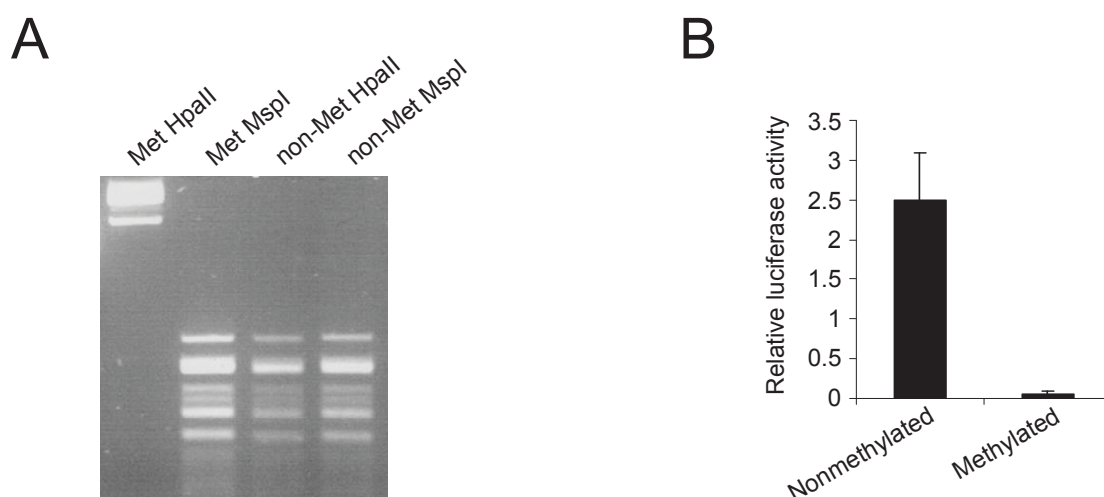
**Figure 35: Demethylation of *Gata1* and *Cd48* promoters by 5-Aza-dC treatment**

Quantitative MassARRAY technology of *Gata1* and *Cd48* promoter sequences revealed partial demethylation 5-Aza-dC treatment in 416B and PU1null cells. Percentages of methylated CpGs are indicated.

### 3.4.3 *Gata1* promoter activity is methylation-dependent

Next, I investigated the causal link between reduction of promoter CpG methylation and differentiation factor gene activation in more detail at the example of *Gata1*. For this purpose I applied a CpG-free luciferase construct generated in Michael Rehli's laboratory [Klug and Rehli, 2006]. This vector allows to determine the effect of promoter methylation on reporter gene expression as only the inserted promoter regions contain CpG sites. Consequently, any effect based on DNA methylation is due to methylation changes in the investigated region. The *Gata1* promoter region, previously described by Sergio Ottolenghi's group [Nicolis, et al., 1991], contains 15 CpG sites and the respective sequence was cloned in front of the luciferase reporter gene. The generated *Gata1*-luciferase construct was *in vitro* methylated using the methyltransferase Sss1 and providing the methyl group donor S-adenosylmethionine (SAM) to the buffer. To test for successful methylation through Sss1, a CpG containing control plasmid, *MSCV-IRES-GFP*, was processed in parallel. This plasmid is equipped with several restriction sites for the enzymes Hpa II and Msp I. While Msp I cuts DNA at its recognition sequence independent of the methylation status of the CpG within its recognition sequence, the methylation sensitive isoschizomere Hpa II cuts only in case of a not methylated CpG. The methylation sensitivity of this restriction enzyme system was used to test whether the control plasmid was successfully *in vitro* methylated. **Figure 36A** illustrates that the non-

methyated construct is equally digested by both enzymes. If, however, the plasmid DNA is methylated, the methylation-sensitive Hpa II fails to cut the vector DNA. Having successfully tested the *in vitro* methylation system, the *Gata1* promoter reporter construct was *in vitro* methylated and luciferase activity was measured after transfection into K562 cells. Luciferase activity was drastically reduced if the *Gata1* promoter sequence was methylated compared to the unmethylated control plasmid. These data suggest that demethylation increases *Gata1* promoter activity providing a causal link between the promoter methylation status and gene activity in case of *Gata1*.



**Figure 36: *Gata1* promoter methylation inhibits reporter gene activity**

**A)** MSCV-IRES-GFP vector DNA was *in vitro* methylated by Sss I in the presence of S-adenosylmethionine (SAM). In non-methylated samples Sss I treatment was performed in the absence of SAM. Digestion with Hpa II or Msp I demonstrated successful *in vitro* methylation. **B)** Luciferase assay of K562 cells transfected with *in vitro* methylated or unmethylated pCpGL-*Gata1* promoter–firefly luciferase construct. Firefly luciferase activity was normalized using *Renilla* luciferase activity as internal transfection control. Values are mean ± s.d..

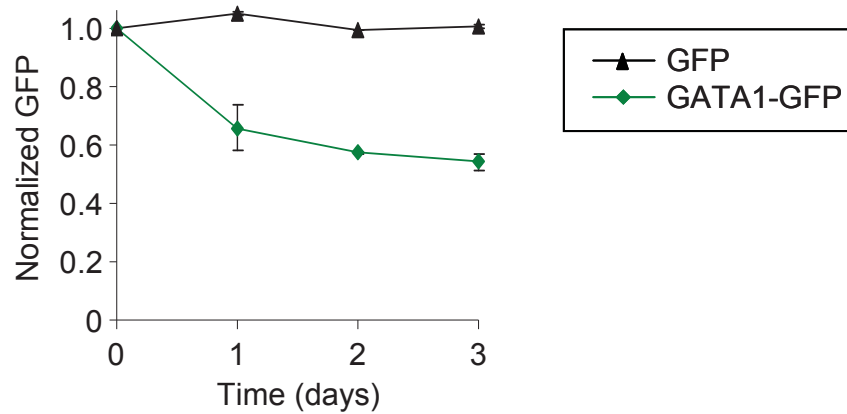
#### 3.4.4 Ectopic *Gata1* expression impairs leukemia cell growth

The results presented in the two previous sections demonstrated that hypomethylation can induce expression of differentiation genes such as *Gata1* in leukemic cells. However, if such gene activation is of functional importance to leukemia cell growth is unclear. To address this question, I tested whether forced expression of *Gata1* affects leukemia cell growth by retrovirally introducing *Gata1* in



Pu1null cells. Ectopic expression of *Gata1* strongly reduced growth of Pu1null cells compared to control cells (**Figure 37**).

Taken together, demethylation induced activation of differentiation factor expression displays a valid mechanism contributing to impairment of leukemia cell function.



**Figure 37: Leukemia cell growth is impaired by ectopic *Gata1* expression**

Enforced *Gata1* expression reduces growth of myeloid leukemia cells. PU1null cells were transduced with retrovirus expressing GATA1 and GFP, or GFP only, and subjected to liquid culture. Percentage of GFP<sup>+</sup> cells were evaluated by FACS over 4 consecutive days. Percentage of GFP<sup>+</sup> cells on day 0 was equalized to 1. Values are mean  $\pm$  s.d..

## 4 Discussion

The aim of this thesis was to investigate how DNA methylation is involved in leukemia stem cell (LSC) function. Constitutive methylation has been shown to be crucial for the maintenance of hematopoietic stem cell (HSC) multipotency. Furthermore, aberrant DNA methylation is a hallmark of cancer. However, so far a functional link between DNA methylation and the establishment or maintenance of unique cancer stem cell (CSC) functions, such as unrestricted self-renewal and, in some cases, lineage pathway choice, was missing. In order to investigate this link, hypomethylated hematopoietic cells were tested in three different oncogene driven leukemia models and consequences of hypomethylation on LSC self-renewal and lineage pathway choice were investigated.

### 4.1 The role of DNA methylation in LSC self-renewal and lineage pathway choices

To study the role of DNA methylation in LSC fate decisions, I chose a knock-down mouse model of the maintenance methyltransferase *Dnmt1*. Other than the methyltransferases *Dnmt3a* and *Dnmt3b*, which act as de novo methyltransferases and are consequently most important for early developmental stages, *Dnmt1* is the main methyltransferase in somatic cells. For this reason, *Dnmt1* was the methyltransferase chosen to be manipulated in the investigations of this thesis. However, it should be mentioned that a recent study showed that *Dnmt3a* mutations frequently occur in myeloid leukemia, suggested that *Dnmt3a* might also play a role in leukemia development [Ley, et al., 2010]. A knockdown approach was applied to test *Dnmt1* function in leukemia, as a complete knock-out of *Dnmt1* was shown to be fatal based on preliminary results from our group (C. Langhans, L. Vockentanz and F. Rosenbauer, unpublished results) as well as previous publications.

#### 4.1.1 DNA methylation critically determines lineage decisions of leukemia initiating cells

The first question addressed in this thesis was as to whether lineage fate decision of leukemia initiating cells are affected by alterations of *Dnmt1* levels. In order to test

this, I used a leukemia model encompassing both acute myeloid and lymphoid leukemia. This was achieved by co-expression of the oncogene *c-Myc* and the anti-apoptotic factor *Bcl2*, which has been shown to induce a bilinear myeloid/B-lymphoid leukemia in mice [Luo, et al., 2005]. *Myc-Bcl2* co-expression achieved by retroviral gene transfer, as expected, created a bilinear leukemia with characteristics about identical to those described by the authors. In sharp contrast, however, *Myc-Bcl2* expressing hypomethylated cells gave rise to a purely myeloid disease. Absence of B-lymphoid leukemic cells was shown by FACS and morphological analysis. This was further confirmed by an exclusively myeloid-specific gene expression pattern compared to a mixed B-lymphoid/myeloid expression pattern observed in bilinear leukemias. Consequently, these data suggested that lineage choices in a bi-potential leukemia system are determined by DNA methylation levels. Any lineage bias based on the lineage composition of the cell population used for initial transduction were ruled out by choosing not-lineage committed stem cells as a starting cell population.

Whereas malignant B-cell development was found to be entirely blocked by low DNA methylation levels, hypomethylated leukemia cells occasionally 'escaped' into an alternative T-lymphoid fate as rare cases of mixed T-lymphoid/myeloid leukemias were observed. T-lineage affiliation of these cells was confirmed by a rearranged T-cell receptor locus. A certain bias of hypomethylated hematopoietic cells towards T-lymphoid neoplasia development was already shown in the publication originally describing *Dnmt1*<sup>-*chip*</sup> knock-down mice. About 80% of *Dnmt1*<sup>-*chip*</sup> mice developed aggressive thymic lymphomas at four to eight months of age [Gaudet, et al., 2003]. However, even though we did not perform large-cohort investigations of aged *Dnmt1*<sup>-*chip*</sup> mice to specifically challenge or reproduce these results, we observed only very rare cases of leukemia development in up to one year old *Dnmt1* hypomorphic mice. Phenotypic characterization of such individual cases rather revealed an acute myeloid leukemia (AML)-like phenotype instead of the occurrence of T-cell lymphomas (data not shown).

Given this ambivalent picture of whether hypomethylation allows or even promotes T-lineage malignancies, I specifically tackled this question applying an oncogene driven T-lymphoma model. *Notch1-IC*, the constitutively active version of the key T-cell factor *Notch1*, was previously shown to transform hematopoietic cells into T-

lymphoma development [Allman, et al., 2001; Pear, et al., 1996]. The transforming nature of *Notch1-IC* was successfully recapitulated in *Dnmt1*<sup>+/+</sup> cells, which gave rise to aggressive T-cell lymphomas. On the other hand, *Dnmt1*<sup>-/-chip</sup> cells with constitutive *Notch1* signaling were unable to form T-ALL. These data suggest that, at least in case of *Notch1-IC* driven T-cell leukemia, hypomethylation blocked the onset T-lineage malignancies. The observed discrepancy between a complete block of T-ALL in the *Notch1-IC* model and the occasional development of T-ALL in the *Myc/Bcl2* model can only be subject to speculations. One possibility could be that, as *Myc/Bcl2* is able to transform hypomethylated cells and create a leukemia, this model harbors a fully transformed leukemia initiating cell which might follow a T-lymphoid differentiation branch later on. However, in the *Notch1-IC* model a more T-cell specific transformation stimulus is offered to the cells and hypomethylated cells might not be susceptible to this in the first place.

Taken together, DNA methylation appears to critically determine lineage fate decisions of leukemia stem/initiating cells. These data suggest that whereas malignant myeloid pathway choice is compatible with low DNA methylation levels, the development of lymphoid leukemias is abrogated in a hypomethylated setting. These observations are clearly due to cell-intrinsic hypomethylation effects as results were obtained from transplantation assays which provide equal wildtype-methylated micro-environments. Cell intrinsic lineage restrictions due to hypomethylation were previously described in physiological hematopoiesis. Reduced *Dnmt1* expression levels in HSCs were demonstrated to cause a block of the lymphoid differentiation branch including both B- and T-lymphoid cells [Broske, et al., 2009]. Additionally, treatment of B-cell leukemia cell lines with the demethylating agent 5-Aza-dC and the histone deacetylase inhibitor trichostatin A (TSA) were shown to cause a complete extinction of the B-cell specific expression program [Ehlers, et al., 2008]. These data support the fact that DNA methylation is indispensable for leukemic B-cell identity.

As described in chapter 1.5.2, DNA methyltransferase inhibitors such as 5-Azacytidine or 5-Aza-2'deoxyctidine are used in the treatment of hematopoietic malignancies. In particular, such agents are approved or in trials mainly for therapy of myeloid malignancies including myeloid dysplastic syndrome (MDS) and AML [Fenaux, 2005; Gore, 2005]. Based on the presented data, such applications might

also be promising for treatment of lymphoid malignancies, especially B-cell leukemias, as DNA methylation was shown to be essential for the development of these diseases. However, we have shown in previous experiments that *Dnmt1* is no longer required for B-cell differentiation once the B-cell program has been established, which is in clear contrast to the evident necessity of DNA methylation for the launch of B-cell commitment at the stem cell level [Broske, et al., 2009]. Consequently, acute B-lymphoid leukemia (B-ALL) might not present a valid target for demethylating therapy. The question whether maintenance of B-ALL, similar to its initiation, relies on DNA methylation, displays an important issue, which should be addressed in future projects. However, an important fact which would have to be taken into account when conducting such investigations is the following: It has been shown that, depending on the nature of the transforming event for B-ALL induction (i.e. the choice of oncogene), the leukemia initiating stem cell origins from different stages of hematopoietic development, including HSCs, B-cell progenitors and committed B-cell progenitors [Castor, et al., 2005]. Thus, LSCs might very differently depend on DNA methylation to retain their stem cell functions. Consequently, demethylating therapy might be varyingly effective depending on the initial transforming event and thus on the nature of the leukemia initiating cell.

#### **4.1.2 DNA methylation is required for proper LSC renewal**

To address the question whether self-renewal capacities of LSCs are affected by DNA methylation levels, I applied an acute myeloid leukemia (AML) model achieved by forced expression of the oncogenic fusion gene *MLL-AF9*. First, I conducted *in vitro* colony-formation assays, which allow a first estimation of leukemic transformability and self-renewal potential. *MLL-AF9* transduced hypomethylated cells showed severely reduced replating capacity compared to *MLL-AF9* transduced wildtype cells. This data allowed the assumptions that reduced levels of DNA methylation diminish the capacity of hematopoietic cells to be immortalized by the *MLL-AF9* oncogene and to (re)gain unrestricted self-renewal reflected by unlimited replating capacity.

To test this assumption in an *in vivo* experiment, *MLL-AF9* driven AML development was investigated in an inducible *Dnmt1* knockdown model, which allowed to

circumvent any experimental bias due to unequal engraftment capacities of hypomethylated cells. AML developed from transformed hypomethylated cells and, whereas the phenotype of these leukemias was unchanged compared to control leukemias, they displayed drastic differences in disease latencies, which confirmed results gathered in the replating assays. Furthermore, limiting dilution experiments were conducted to specifically test self-renewal capacities of *Dnmt1*<sup>Δ/chip</sup> leukemia cells. Control leukemias (*Dnmt1*<sup>lox/chip</sup>) were determined to possess an LSC frequency of 1 in 91. These results were in line with data published by Scott Armstrong's group, as based on their results, obtained in limiting dilution experiments with *MLL-AF9* leukemic bone marrow cells, an LSC frequency of 1 in 126 could be calculated [Krivtsov, et al., 2006]. In contrast to the number calculated from control leukemias, hypomethylated leukemias displayed an LSC frequency of 1 in 1,072 cells. Consequently, *Dnmt1* hypomorphic leukemias revealed an 11.8-fold decrease in cells, which are able to initiate leukemia in a secondary recipient thus, by definition, display functional LSC. The fact that prolonged latencies were indeed caused by less functional LSCs was further underlined by the observation that hypomethylated *MLL-AF9* blasts displayed equal proliferation rates during *in vitro* culture compared to wildtype counterparts suggesting that leukemia bulk growth is rather unaffected by DNA hypomethylation.

Non-inducible, direct knockdown (*Dnmt1*<sup>-/chip</sup>) *MLL-AF9* leukemias were generated for further experiments which aimed at the more detailed analysis of the LSC compartment. I reasoned this switch of mouse models necessary as few leukemic cells which had escaped cre-mediated *Dnmt1* depletion overgrew hypomethylated leukemias due to selection advantages. Confirming previous results presented here, development of *Dnmt1*<sup>-/chip</sup> leukemias was again marked by significantly prolonged latency times. An LSC-enriched cell population, defined by strong expression of the surface marker c-Kit was isolated from leukemias according to previous descriptions [Somervaille, et al., 2009]. Enrichment of cells with LSC identity in the c-Kit<sup>high</sup> cells was confirmed in both hypomethylated as well as in control leukemias by clearly enhanced colony-forming ability as compared to c-Kit non-expressing cells. Functional testing of leukemic self-renewal of hypomethylated c-Kit<sup>high</sup> cells in serial replating assays revealed a progressive loss of cells with self-renewal potential within this cell pool. In contrast to that, wildtype c-Kit<sup>high</sup> cells were able to maintain a

constant level of self-renewing colony-forming cells.

These results were furthermore underlined by *in vivo* experiments. To compare the leukemia initiating potential of *Dnmt1*<sup>-chip</sup> and *Dnmt1*<sup>+/+</sup> *MLL-AF9* c-Kit<sup>high</sup> cells these cells were subjected to a limiting dilution experiment. With a calculated LSC frequency of 1 in 33 in *Dnmt1*<sup>+/+</sup> c-Kit<sup>high</sup> cells, the frequency of cells capable of initiating a leukemia was about three-fold increased in the LSC-enriched cell population. *Dnmt1*<sup>-chip</sup> c-Kit<sup>high</sup> cells, however, displayed an LSC frequency of 1 in 478, confirming a clearly diminished number of LSCs due to hypomethylation.

Importantly, colony numbers in serial replating assays were comparable on the first plate where freshly isolated, ex-vivo c-Kit<sup>high</sup> leukemia cells were seeded. These data would argue that the number of colony forming cells in a terminal leukemia, being a measure for the LSC frequency, is actually similar, as it has been shown previously that expanded cell pools originating from a single colony are able to initiate leukemia development after transplantation [Somervaille and Cleary, 2006]. Consequently, one might speculate that hypomethylated leukemias might actually possess a comparable number of LSCs, which, however, are deficient in self-renewal capacity accounting for drastically diminished leukemia-forming capacity. To investigate this further, one could expand single *Dnmt1*<sup>-chip</sup> colonies and test whether these cells are able to form leukemia upon transplantation. If so, this would point to the conclusion that decreased leukemia formation due to hypomethylation is caused by restricted self-renewal and/or proliferative capacities of individual transplanted LSCs in the recipient.

#### **4.1.3 Hypomethylated bone marrow environment does not affect leukemia development**

Contrasting the severe cell intrinsic effects of DNA methylation on LSC functions, the methylation status of the bone marrow microenvironment was shown not to be critical for *MLL-AF9* LSC functioning. Wildtype leukemias developed equally, both regarding phenotype as well as leukemia-latency, independent of being surrounded by hypomethylated and physiologically methylated stroma cells. This issue was important to be investigated as hypomethylation effects of bone marrow stroma cells on leukemia cells have to be thoroughly taken into account regarding the



therapeutical use of DNMT inhibitors, as the microenvironment is equally exposed to these drugs as the hematopoietic/ leukemic cells. Moreover, cell-to-stroma interactions have been shown to be crucial for the development of leukemia. The phenotype of human *MLL-AF9* leukemia cells was shown to be dependent on the stroma-cell cytokine-producing characteristics of the recipient mice [Wei, et al., 2008]. The absence of hypomethylated niche effects on leukemia fates presented here are in line with data from experiments conducted in our lab concerning the impact of reduced DNA methylation on normal hematopoiesis. We found that wildtype hematopoietic donor cells equally repopulated both *Dnmt1*-knockdown and *Dnmt1*<sup>+/+</sup> recipient mice both in regard to general engraftment capacities as well as lineage composition (unpublished data). In conclusion, whereas both leukemic as well as hematopoietic stem cells are greatly impacted by cell-intrinsic loss of DNA methylation, no obvious effects on their function could be observed as a consequence to microenvironmental hypomethylation.

#### **4.1.4 Differentiation factors induced by pharmacological demethylation inhibit leukemia growth**

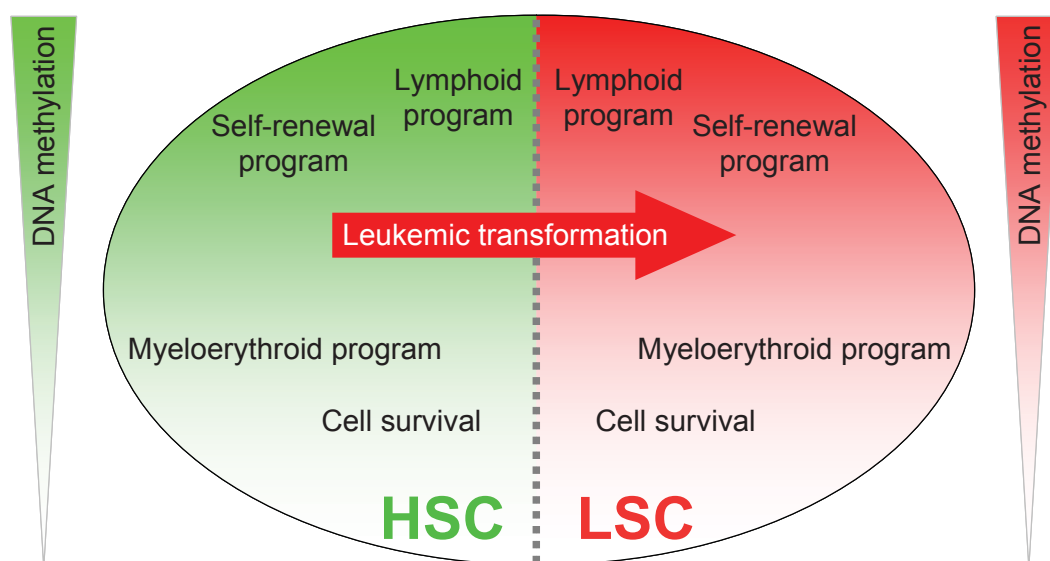
Studies on the role of DNMT1 in hematopoiesis using a genetic knockdown of *Dnmt1* (*Dnmt1*<sup>-/-chip</sup>) revealed that hypomethylated HSCs display an altered gene expression pattern, which is more akin to a myeloerythroid progenitor signature rather than a stem cell signature. This up-regulation of myeloerythroid-specific factors, which HSCs are usually devoid of, was shown to result in impaired stem cell self-renewal [Broske, et al., 2009]. These observations raised the possibility that treatment of cancer cells with demethylating agents might be able to activate expression of differentiation factors, which might ultimately lead to a reduction of malignant growth. Additionally supportive of this hypothesis is the fact that hematopoietic differentiation factors such as *Id2* and *Cebpa* had earlier been shown to be silenced by methylation in cancer cells [Flotho, et al., 2009; Hackanson, et al., 2008]. Indeed, I could show that 5-Aza-dC treatment of different myeloid cell lines and primary leukemia cells was followed by an up-regulation of the differentiation factors *Gata1* and *CD48*. The observed up-regulation was paralleled by a loss of promoter methylation at the respective gene loci. Thus, demethylating agents are able to revert the DNA methylation mediated gene silencing of factors repressed in cancer cells. A direct link to DNA methylation

dependent gene regulation was shown at the example of *Gata1*. Methylation of the region upstream of the *Gata1* transcriptional start site was shown to drastically reduce gene expression in a luciferase reporter assay compared to the non-methylated upstream region. Biological significance for enhanced differentiation factor expression on leukemic proliferation was directly proven by ectopic expression of *Gata1* in transformed myeloid Pu1null cells. Reduced cell growth in *Gata1* expressing cells compared to control cells suggested that induced differentiation by these factors interferes with leukemic cell renewal and/or proliferation. Taken together, pharmacological demethylation is able to counteract the methylation mediated gene silencing of differentiation factors. Elevated expression of such factors, in turn, inhibits neoplastic growth, consequently displaying an interesting approach to interfere with leukemia stem cell self-renewal.

DNA methylation is known to be tightly interconnected with histone modification mechanisms. This interconnectedness is known to act in a bi-directional manner. For example *de novo* DNA methyltransferases are known to be specifically recruited to loci which already display repressive histone marks. On the other hand it has been shown that histone modifying enzymes are recruited to DNA methylated loci [Cedar and Bergman, 2009]. Consequently, it might be interesting to thoroughly investigate DNA (de-)methylation dependent histone modifications occurring in cancer cells. Detailed understanding of epigenetic events in cancer cells can be of great value in the development of more advanced epigenetics treatment strategies.

## 4.2 Conclusions and model

Summarizing the results presented in this thesis, I propose a model depicted in **Figure 38**, which illustrates the role of DNA methylation for LSC function and multipotency. This model suggests that normal stem cells, here represented by HSCs, and cancer stem cells, here represented by LSCs, share similar methylation dependent control mechanisms to exercise common functional properties [Broske, et al., 2009; Vockentanz, et al., 2010].



**Figure 38: Distinct DNA methylation dosage requirements of leukemia stem cells**

Leukemia stem cells (LSCs) display specific DNA methylation dosage requirements for different cellular programs, which is in analogy to the role of DNA methylation in hematopoietic stem cell (HSC) function. Leukemic stem cell self-renewal and the establishment of a neoplastic lymphoid pathway program depend on high levels of DNA methylation. Myeloerythroid programming of LSCs and HSCs, on the other hand, is feasible with low cellular dosages of DNA methylation. A certain minimal level of DNA methylation, however, is required to prevent apoptosis in HSCs and assumingly also in LSCs. Leukemic transformation, the process of equipping (stem) cells with uncontrolled self-renewal capacities, relies on high levels of DNA methylation [Vockentanz, et al., 2010].

Specifically, I found that certain programs in the functional repertoire of LSCs rely on high levels of DNA methylation to be properly fulfilled. This includes the initial transformation process, the maintenance of malignant stem cell self-renewal as well as commitment to the lymphoid lineage. Similar requirements have been stated for HSC self-renewal and lymphoid pathway choice [Broske, et al., 2009]. A further parallel is found in the fact that low DNA methylation levels suffice for myeloerythroid programming. Moreover, first results (not shown here) and previously published data hint towards the fact that a minimal level of DNA methylation is indispensable for LSCs survival as it has been shown HSC survival. Additionally, the premature activation of myeloerythroid differentiation factors due to missing methylation mediated gene silencing, which was found to be responsible for restricted capacities of hypomethylated HSCs, was demonstrated to display a valid mode of action to

inhibit malignant growth by pharmacological demethylation.

In summary, the experiments presented in this thesis demonstrated that DNA methylation is critical for the capacity of LSCs to exert multi-lineage fate programs, which raises the hypothesis that retention or re-establishment of stem cell-specific methylation patterns may be an important step in the development and function of LSCs.

### **4.3 Perspectives**

The results gathered in this thesis provide a general picture of the role of DNA methylation in CSCs or, more specifically, in LSCs. However, it is still unresolved which gene programs promoting leukemic self-renewal are controlled by DNA methylation. This question is subject of a future project in this group, which aims at pinpointing epigenetically regulated genes which are crucial for cancer self-renewal. For this purpose LSCs from hypomethylated and control leukemias will be analyzed by global mRNA sequencing. This method will allow an in depth insight into the transcriptional regulation of LSCs by DNA methylation. Besides the crucial information on the methylation dependent regulation of “leukemia self-renewal genes”, this analysis might furthermore detect new surface markers which are present on hypomethylated stem cells. Such a discovery would be of great interest from a therapeutical point of view, as surface markers are a valuable target for anti-cancer therapy by therapeutic antibodies. A surface marker present on hypomethylated tumor stem cells but not on the normal tissue stem cells might be a promising approach for a combinatorial cancer treatment encompassing demethylating drugs and specifically tailored antibody therapy.

In conclusion, for development of novel therapeutic strategies specifically targeting cancer stem cells, it is inevitable to understand the molecular basics of how these cells acquire and maintain unlimited self-renewal potential. As this work demonstrated a crucial role for DNA methylation in these processes, a detailed understanding of methylation mediated control of self-renewal programs displays an important step for the development of cancer stem cell targeted therapy.

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## Abbreviations

% percentage

**Aa** amino acid

**AGM** aorta-gonad-mesonephros region

**ALL** acute lymphoid leukemia

**AML** acute myeloid leukemia

**APC** allophycocyanin or adenomatous polyposis *coli*

**bcl-2** B-cell lymphoma 2

**BCR** B cell receptor

**BM** bone marrow

**bp** base pair

**BSA** bovine serum albumin

**C** cytosine

**CD** cluster of differentiation

**C/EBP** CCAATT/enhancer binding protein

**CLP** common lymphoid progenitor

**CMP** common myeloid progenitor

**csf1r** colony stimulating factor 1 receptor

**c-Myc** myelocytomatosis viral oncogene homolog

**CpG** cytosine guanine dinucleotide

**Cre** cyclization recombination protein

**CSC** cancer stem cell

**Csf2ra** (=Gm-csfr) granulocyte/macrophage stimulating factor receptor

**CTCF** CCCTC-binding factor

**DAPI** 4',6-diamidino-2-phenylindole

**DMEM** Dulbecco's modified Eagle medium

**DMSO** dimethylsulfoxid

**DNA** desoxyribonucleic acid

**dNTP** deoxynucleotide triphosphate

**DTT** dithiotreitol

**E** embryonic day

**e.g.** exempli gratia

**Ebf1** early B cell factor 1

**EDTA** ethylenediaminetetraacetate

**Env** envelope protein

**EPO** erythropoietin

**ES cells** embryonic stem cells

**EZH2** enhancer of zeste homolog 2

**FACS** fluorescent activated cell sorting

**FCS** fetal calf serum  
**FITC** fluorescein isothiocyanate  
**FL** fetal liver  
**Flt3** Fms- related tyrosine kinase 3  
**Flt3L** Flt3 ligand  
**fw** forward  
**g** gravity  
**GMP** granulocyte-macrophage precursor  
**G/M-CSF** granulocyte/macrophage stimulating factor  
**G9A** also known as **EHMT2** euchromatic histone-lysine N-methyltransferase 2  
**Gadd45** growth arrest and DNA damage  
**Gag-pol** group antigen protein - polymerase  
**Gata1** GATA binding protein 1 or globin transcription factor 1  
**GFP** green-fluorescent protein  
**Gy** gray  
**h** hour  
**HDAC** histone deacetylase  
**HP1** heterochromatin protein 1  
**HSC** hematopoietic stem cell  
**ICF** immunodeficiency, centromeric instability and facial anomalies syndrome  
**Ig** immunoglobulin  
**Igf2** insulin-like growth factor 2  
**IL** interleukin  
**IRES** internal ribosomal entry site  
**Kb** kilobase  
**KCl** potassium chloride  
**KH<sub>2</sub>PO<sub>4</sub>** potassium phosphate monobasic  
**Lif** leukemia inducing factor  
**Lin** lineage  
**LN** lymph node  
**LOH** loss of heterozygosity  
**LSC** leukemia stem cell  
**M** molar  
**MBP** Methyl-CpG-binding proteins  
**MBD** Methyl-CpG-binding domain  
**MDS** myelodysplastic syndrome  
**MEM** modified Eagle's Medium  
**MEP** megakaryocyte-erythrocyte precursor  
**mg** milligram  
**MIG** MSCV-IRES-GFP  
**min** minutes



**Mk** megakaryocyte/ megakaryocytic  
**ml** millilitre  
**MLL** mixed lineage leukemia  
**mM** milli molar  
**µg** microgram  
**µl** microliter  
**µM** micromolar  
**MP** myeloid progenitor  
**MPP** multi-potent progenitor  
**mRNA** messenger ribonucleic acid  
**Mx1** myxovirus (influenza virus) resistance 1  
**n** number  
**Na<sub>2</sub>HPO<sub>4</sub>** Sodium phosphate dibasic  
**NaCl** Sodiumchloride  
**Nk** natural killer  
**NLS** nuclear localization sequence  
**P** probability value  
**Pax5** paired box gene 5  
**PBS** phosphate buffered saline  
**PcG** polycomb group  
**PCR** polymerase-chain-reaction  
**PE** Phycoerythrin  
**PI** propidium iodide  
**Pol** polymerase  
**Polybrene** hexadimethrine bromide  
**pSP** para-aorta splanchnopleura  
**P/S** penicillin/streptomycin  
**rev** reverse  
**RNA** ribonucleic acid  
**rpm** rotations per minute  
**RT** room temperature  
**RT-PCR** reverse transcription real time polymerase chain reaction  
**s.d.** standard deviation  
**SAM** S-adenosylmethionin  
**SCF** stem cell factor  
**SDS** Sodiumdodecylsulfat  
**T** thymidine  
**T4** T4 DNA polymerase  
**TAE** Tris/acetate/EDTA buffer  
**Taq** Taqman DNA polymerase  
**TE** Tris/EDTA

**TPO** thrombopoietin  
**TSG** tumor suppressor gene  
**tRNA** transfer RNA  
**UV** Ultraviolet  
**V** Volt  
**WT** wild type  
**X** X chromosome  
**YFP** enhanced yellow fluorescent protein  
**YS** yolk sac

# Selbständigkeitserklärung

Ich versichere hiermit, dass die von mir vorgelegte Dissertation eigenständig und ohne Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe. Ich versichere, dass alle aus anderen Quellen oder indirekt übernommenen Daten und Konzepte, sowie Ergebnisse aus Kooperationsprojekten unter Angabe der Referenz gekennzeichnet sind.

Außerdem versichere ich, dass mir die aktuelle Promotionsordnung bekannt ist und ich mich nicht anderwärts um einen Doktorgrad bewerbe, bzw. noch keinen entsprechenden Doktorgrad besitze.

Berlin, den 28. Januar 2011

Lena Vockentanz

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